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Phytophthora Sojae Infecting Soybean: Pathotype Diversity, New Sources of Resistance and Interaction with the Soybean Cyst Nematode

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PHYTOPHTHORA SOJAE INFECTING SOYBEAN:

PATHOTYPE DIVERSITY, NEW SOURCES OF RESISTANCE AND INTERACTION WITH THE SOYBEAN CYST NEMATODE

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Plant Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of major department.

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I would like to dedicate this thesis to my family; my mother Bilquis Alam Chowdhury, my father Raisul Alam Chowdhury, my sister Reema Najma Chowdhury and my brother Late Arif Alam Chowdhury. They have always encouraged me to pursue my passions. I am forever grateful for their never-ending love and support.

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ABSTRACT

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Phytophthora root and stem rot, caused by *Phytophthora sojae* Kaufmann and Gerdemann, is an important disease of soybean (*Glycine max* L.) in South Dakota. To gain a better understanding of the importance of *P. sojae* in South Dakota, specifically pathotype diversity, identification of new resistance sources and the interaction with the soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN), this research was undertaken with the following objectives - 1) to characterize the pathotype diversity of *P. sojae* causing Phytophthora root and stem rot on soybean in commercial fields in South Dakota; 2) to compare inoculation methods to evaluate for partial resistance to *P. sojae* on soybean and identify new sources of resistance to two virulence pathotypes of *P. sojae* in a recombinant inbred line (RILs) population derived from the cross between cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916); and 3) to study the interaction between SCN and *P. sojae* on soybean. In order to achieve the objectives, a total of 114 isolates of *P. sojae* were recovered from soil samples covering 30 counties in South Dakota during a three year survey (2013 - 2015), of which 70 *P. sojae* isolates were pathotyped using 13 standard soybean differentials. Results suggest that mean complexity of the *P. sojae* pathotypes have increased over time and over 85% of the *P. sojae* isolates were able to defeat *Rps*1a, *Rps*1c and *Rps*1k that are commonly deployed *Rps* genes in the commercial

cultivars of South Dakota. In order to find new sources of partial resistance to *P. sojae*, a qualitative comparison among three inoculation methods (inoculum layer test, tray test and rice grain inoculation) was accomplished in the greenhouse. Based on the recovery of *P. sojae* isolates (%), inoculum layer method was adopted to screen 100 recombinant inbred line (RIL) for partial resistance to two virulence pathotypes of *P. sojae* identified in South Dakota [PS-15-TF3 that is virulent on all 13 soybean differentials and PS-14-F14 that is virulent on only one differential (*Rps*7)]. As compared to the parents of the RIL population, [*Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916)] we found 9 RILs that had relatively shorter lesion length (0 to 5 mm) when inoculated with either of the *P. sojae* isolates. To study the interaction between SCN and *P. sojae* on soybean, a greenhouse experiment was set up in a completely randomized design in a factorial arrangement with four soybean cultivars (Jack, Surge, William 82 and Williams). Two isolates of *P. sojae* representing two different virulent pathotypes (PS-15-TF3 and PS-14-F14) and SCN HG-type 0 representing the most commonly found HG-type in South Dakota was used to perform inoculations. For all the cultivars, we observed that the lesion length was caused by *P. sojae* was increased in the presence of SCN relative to *P. sojae* treatment. However, SCN population was reduced in the presence of both the pathogens. The findings of our study highlight the high pathotype diversity of *P. sojae* and and increased lesion size when *P. sojae* co-infects with SCN. This information will help with the development of effective and improved strategies for managing Phytophthora root and stem rot through deployment of resistant genes in commercial soybean varieties that are likely to be more durable, managing SCN to reduce severity of Phytophthora root rot, and incorporation of identified resistance to *P. sojae* in RIL population for future breeding efforts.

CHAPTER 1

General Introduction

The host soybean

Soybean, *Glycine max* L (Merr.), belongs to the family *Fabaceae* (*Phaseoleae*) and is a type of legume which is native to East Asia. Until after the Chinese-Japanese war (1894 to 1895), soybean production was only concentrated in China (Hartman et al. 1999). During 1908, soybeans were imported to Europe as soybean oil cake for using it as a fertilizer and since then soybean gathered worldwide attraction. It is believed that soybean was introduced to the American colonies as "Chinese vetches" during the year 1765 by Samuel Bowen, a sailor who had visited China. During 1879, the Rutgers Agricultural College in New Jersey started the testing of soybeans in a scientific agricultural school in the United States. Soybean was continued to be popular in the eastern and southeastern United States for several years. After the World War II, soybean production was moved from the southern United States into the Corn Belt (Hartman et al. 1999). Currently, soybean is considered to be the top oilseed crop produced and consumed in the world. In 2015-2016, around 320 million metric tons of soybeans were produced worldwide (https://www.statista.com/statistics/267271/worldwide-oilseed-production-since-2008/).

Although soybean is grown in more than 50 countries in the world, the United States is considered the world's leading producer since the past half century (Wilcox 2004). The United States accounts for 34 percent of the world's soybean production and according to the commodity basis, United States is also the largest exporter of raw soybeans (42 percent market share) (Wrather et al. 1997). In the United States, there are around 34.4

million hectares which are used for planting of soybeans. During 2009 soybean production was 93 thousand metric tonnes, which valued for around $31 billion (NASS 2012). From 2014-15, the annual production of soybeans in the three seasons ranged between 82.8 and 108 million metric tons (http://www.worldatlas.com/articles/world-leaders-in-soya-soybean-production-by-country.html).

In general, the main challenge in crop production systems is to reduce the impact of plant pathogens and other pests on the crop yield. On soybean, yield suppression due to individual diseases varied among the regions and years in the United States. For example, during 2003 to 2005, soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN) suppressed soybean yield more than any other diseases in the United States followed by Phytophthora root and stem rot, sudden death syndrome, and other soybean seedling diseases (Wrather and Koenning 2006).

The pathogen *Phytophthora sojae*

The oomycete *Phytophthora sojae* Kaufmann and Gerdemann (syn. *Phytophthora megasperma* f. sp. *glycinea* Kuan and Erwin) belongs to the family *Pythiaceae* and kingdom *Stramenopila* (Brasier 1992; Hansen and Maxwell 1991). Oomycetes are more closely related to heterokont algae (brown and golden brown algae) and diatoms, than to true fungi (Brasier 1992). As like other oomycetes, species of *Phytophthora* possess biflagellate zoospores, alga-like gametangia, glucans and cellulose containing cell walls, and diploid vegetative cells (Hardham 2009). Based on morphology, *Phytophthora* has some resemblance with fungal pathogens; for example, *Phytophthora* produces thread like structures called mycelium (Beakes and Sekimoto 2009). Nevertheless, many physiological traits differ *Phytophthora* and other oomycetes from true fungi, because of

which they are classified into a separate kingdom (Beakes and Sekimoto 2009). Unlike true fungi, oomycetes are more adapted to aquatic habitat. Fungi has chitin in their cell wall whereas, oomycetes cell wall is composed of glucan and cellulose. Oomycetes have coenocytic mycelium which lacks septation or division contrasting fungi (Beakes and Sekimoto 2009). As opposed to haploid true fungi, oomycetes have diploid vegetative stage. Anton de Bary who described the potato late blight pathogen in 1876, gave the genus name *Phytophthora* which means 'plant destroyer' (Schumann et al. 2000). Most species of *Phytophthora* have sexual life cycle that produces sexual structure called oospore. Oospores are usually thick walled and resistant to extreme environmental conditions (Judelson 2009). There are also some species in the groups that are heterothallic (cross-fertile) and they require two mating (compatible) types to produce oospores. Oospores have very long longevity, at least for months (Pittis et al. 1994) and possibly for years in soil (Duncan et al. 1980).

Besides sexual oospores, asexual propagules are also developed on the host tissue. Some species of *Phytophthora* possess detached (caducous) sporangia which are adapted for aerial dispersal over long distances (Hardham 2009). Few species of *Phytophthora* also have non caducous (do not shed or break off from main mycelium) type of sporangia that can spread in water (Hardham 2009). In free moisture conditions, the biflagellate swimming spores are also released from the sporangia, which usually are chemotactic, thus can perceive and swim towards suitable hosts (Hardham et al. 1991).

In 1963, Waterhouse subdivided genus *Phytophthora* into six groups. *Phytophthora megasperma* var *sojae*, which is the currently known as *P. sojae*, was placed in group V (Erwin et al. 1983). The typical terminal sporangia of *P. sojae* is non-papillate and ellipsoid

or obpyriform that ranges from 23 to 88 x 16 to 52 μm (Erwin et al. 1996). *Phytophthora sojae* has a globose shaped oogonium (female structure) with more than 40 μm in diameter and antheridia (male structure) are mainly paragynous (attach to the oogonial stalk) but can also be amphigynous (surrounded the oogonial stalk) (Dorrance et al. 2007). The optimal temperature for formation and germination of oospores is 24^{o}C (Erwin et al. 1998). In addition to morphology based identification of *P. sojae*, molecular tools have been used to confirm the identity of the pathogen. For example, single-strand conformational polymorphism analysis based on PCR amplified ribosomal DNA internal transcriber spacer 1 have been used for DNA fingerprinting in order for species identification (Gallegly et al. 2008). Moreover, molecular identification of *P. sojae* causing Phytophthora root and stem rot on soybean in Taihe, China was performed by amplifying internal transcribed spacer (ITS) region with ITS4 and ITS6 primers (Dai et al. 2015)

Phytophthora sojae is not the only soybean-infecting species of *Phytophthora*. It has been described by Hamm and Hansen (1981), that there are some isolates in the *P. megasperma* complex that were pathogenic on soybean. Successively, isolates of non-classifiable species of *Phytophthora* were reported in Indiana that cause root rot on soybean (Reeser et al. 1991). In Illinois, an unknown *Phytophthora* sp. that can infect and kill soybeans was detected by Malvick and Grunden (2004). Preliminary comparisons among ITS DNA sequence alignments from Illinois isolates and *Phytophthora sansomeana* Hansen and Reeser holotype indicate that these isolates are very closely related to *P. sansomeana* (Malvick and Grunden 2004). In recent times, these isolates were designated as a new species of *Phytophthora* and was named *P. sansomeana* (Hansen et al. 2009;

Reeser and Hansen 2009). In Ohio, *P. sansomeana* was reported as a pathogen not only of soybean, but also of corn, *Zea mays* (Zelaya Molina et al. 2010).

The disease Phytophthora root and stem rot

Disease symptoms

Symptoms of Phytophthora root and stem rot may differ over the growing season of the year for the reason that the disease can infect soybean at different stages of crop development. Infection may occur from the pathogen after the soybean seeds swell and before germination of the seeds, this stage is called the seed rot phase of the disease (Pre-emergence damping off). Early-season diseases like, seed rot and damping-off are highly favored by the flooded soil conditions within one week of planting. Post-emergence damping off of soybean seedlings may occur showing prompt wilting and plant death, if infection occurs before or within a few days after emergence. Depending on the level of resistance in the cultivar the symptoms of the damping off phase may differ and can range from asymptomatic infection to stunted, chlorotic, and wilting plants (Dorrance et al. 2007). Sometimes dark and discoloration on the stem tissue can also be seen. Starting from July the root and stem rot phase may be visualized. The pathogen invades through the roots and spreads into the lower stem. Brown stem girdling lesions that extended up the plant from below ground level can be seen with the course of disease progress. Leaf wilting may be seen first, then petioles (leaf stems) drooping starting at the older leaves and gradually continuing upward on the stem (Schmitthenner 1985). Healthy plants may grow taller in the later period of the season that can hide the killed plants and as a result, the problem of having Phytophthora root and stem rot may seem less severe.

Disease cycle

Phytophthora sojae overwinters as oospores in soil either in crop residues or exposed to soil after decomposition of the residues and these oospores serves as the primary inoculum for the following growing season (Figure 1.1). Oospores are formed after the fertilization and sexual recombination (meiosis) of male (antheridum) and female gametes (oogonium).The oospores can survive in the extreme soil and environmental conditions as dormant spores for several years as the oospores possess thick cell walls with cellulose (Hartman et al. 1999). Soil temperatures above 15^{o}C is suitable for oospore germination and germination may be delayed if the soil temperature is less than 15^{o}C (Dorrance et al. 2007). Therefore, it is thought that during spring under suitable moisture and temperature conditions, the dormancy of oospores is broken and they begin to germinate and produce sporangia. The oospore can germinate directly as sporangia and penetrate the host cells at the plant's root tip. In case of indirect germination, the sporangia releases zoospores which encyst on the host plant cells and germinate (Tyler et al. 2007). Zoospores are produced when soils are flooded or saturated with water. Zoospores are biflagellate asexual motile spores which can move through water films in the soil and capable to infect the roots of plants or seeds (Figure 1.1). In the saturated soils the zoospores can make short distances upto 1 cm but they mainly spread through moving flood water (Schmitthenner 1999).

The zoospores are attracted by root exudates (Morris et al. 1992), specifically chemicals like, deadzeins and genistein that are released at the tip of the plant roots. They swim to the host root and encyst on the root surface, germinate and penetrate the roots. The pathogen forms an appressorium at the end of germ tube to penetrate into the host tissue. The growth of the hyphae is intercellular in root cells which grow intracellularly in

hypocotyls. Globular and fingerlike haustoria are produced by *P. sojae* to uptake nutrients and colonize the plant (Schmitthenner 1999). Susceptible, tolerant, and resistant cultivars differ with the amount of oogonia and oospore production in infected root and stem tissues. Nevertheless, less oospores are formed in resistant cultivars compared to susceptible and tolerant cultivars (Grau et al. 2004). Leaf infections are rarely seen but can be developed through the splashing of the pathogen on the leaves during rainstorms. Under misty and cloudy weather conditions, severe leaf infection can be seen and the pathogen can spread towards the petioles and stems.

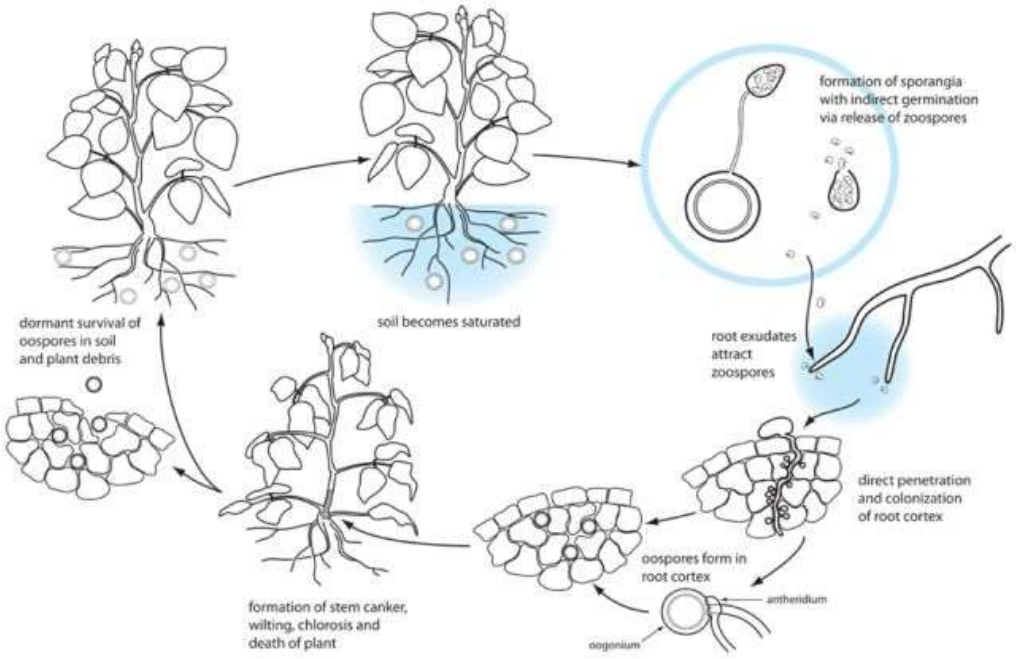

Figure 1.1 Disease cycle of Phytophthora root and stem rot (Dorrance et al. 2007).

Heavy, poorly drained or compacted and fine texture (clay) soil is very common for Phytophthora root and stem rot. *Phytophthora sojae* population densities are higher in no-till areas with fine textured soil as compared to no-till areas with moderately textured soil. For infection of soybean plants by *P. sojae*, the ideal temperature is 15.5 to 26.6^{o}C

Damage caused by this disease may increase by consecutive years of growing soybeans on the same field. Use of excessive levels of potash, manure or municipal sludge just before planting may increase the severity of the disease plant (Schmitthenner 1999).

Management of Phytophthora root and stem rot

Host resistance

To manage Phytophthora root and stem rot of soybean, *R*-gene mediated or race specific resistance, root resistance, and partial resistance have been described. A study was carried out by Slaminko et al. (2010) on 3,533 commercial soybean cultivars used in the United States to assess their resistance to *P. sojae* and they found that 51% of the cultivars carried at least single *Rps* gene. There were 50% cultivars that had *Rps*1c and 40% of the cultivars had *Rps*1k mediated *P sojae* resistance. At this time, among the several types of resistance, soybean varieties with race specific resistance in combination with partial resistance are recommended to manage *P. sojae*.

The soybean genome possess *R*-genes that encode nucleotide binding site-leucine rich repeat (NBS-LRR) type of proteins, which recognize effector proteins of the pathogens to induce defense response. *R*-gene mediated response is usually expressed as hypersensitive response in the host. In the case of *P. sojae*, *Rps* (Resistance to *P. sojae*) genes have been described which is race specific and has provided reasonable protection against the majority of *P. sojae* populations in the United States for the last four decades (Bhattacharyya et al. 2005). A total of 20 *Rps* loci including 25 alleles have been mapped on soybean genome (Demirbas et al. 2001; Fan et al. 2009; Gao et al. 2005; Lin et al. 2013; Sugimoto et al. 2011; Sun et al. 2011; Weng et al. 2001; Wu et al. 2011a; Yao et al. 2010; Zhang et al. 2013; Ping, et.al. 2015). Among the described *Rps* genes, *Rps*1a was the first

resistance gene that was deployed in USA during 1960s and it remained effective for almost eight years (Grau et al. 2004; Schmitthenner 1985). Approximately 5% of the commercial cultivars still have *Rps*1a gene and they are still in use in Midwestern USA (Slaminko et al. 2010). Resistant genes such as *Rps*1c, *Rps*1k, *Rps*3a and *Rps*6 were extensively deployed in the Midwest region of USA (Dorrance et al. 2003; Gordon et al. 2007). However, for the last two decades *Rps*1k has been widely used for its stable performance and conferring resistance against large number of North American *P. sojae* races (Gao et al. 2005; Schmitthenner 1994).

Partial resistance is defined as the ability of susceptible plants to survive in case of infection without showing severe symptoms like death, stunting, or yield loss (Glover and Scott 1998). Dorrance et al. (2003) examined the effect of partial resistance on Phytophthora root rot incidence and seed yield of soybean in Ohio, and concluded that genetic traits that are associated with high levels of partial resistance do not have a negative effect on yield. Walker and Schmitthenner (1984) studied the heritability of tolerance to Phytophthora root rot in soybean and found that race-specific resistance and tolerance were not completely independent. Even though we can use cultivars with partial resistance cultivars in planting, additional control measures such as a combination of race-specific resistance with partial resistance, improved soil drainage, hilled row planting, or seed treatment with a fungicide might be necessary.

Root resistance is a kind of resistance that is quantitatively inherited (several genes that each contribute to the level of resistance) and is considered to be showing complete resistance (Dorrance et al. 2007). Expression of resistance for both root and partial resistance is mainly on roots.

Visual evaluation methods such as hypocotyl inoculation technique is used for screening of race-specific resistance and the inoculum layer test has been used for screening partial resistance. These two techniques have been widely used to evaluate soybeans to identify possible new sources of resistance (Dorrance and Schmitthenner, 2000; Mideros et al. 2007). In the layer test, a specified distance is maintained to place the agar culture of the pathogen below the seed during planting time and incidence and severity of the disease is evaluated 3 to 4 weeks later using a 1 to 10 scale (Dorrance et al. 2006).

Chemical control

In order to reduce the losses due to Phytophthora root and stem rot, seed treatment fungicides such as metalaxyl (Allegiance), oxadixyl (Anchor), and mefanoxam (Apron XL) are highly effective against *P. sojae* and other oomycetes (Draper and Chase 2001). The seed treatments are used for managing early season seed decay and damping-off caused by *P. sojae*. For effective management of *P. sojae*, a higher seed treatment rate is needed than that would be used to control species of *Pythium*, the other causal agents of damping-off of soybean (Dorrance et al. 2007). Under favorable disease environment application of metalaxyl in furrow or as seed treatment has improved plant emergence and yields in susceptible and low partial resistant cultivars (Anderson et al. 1982; Grau et al. 2004; Schmitthenne 1985).

Cultural practices

Areas in the soybean fields that are low lying, prone to flooding or poorly drained are more likely to develop Phytophthora root rot. Therefore, cultural practices related to improved soil drainage contribute to the reduction in the time that soils are saturated and ultimately reduces the *P. sojae* infection period. Soil drainage can be promoted by cultural

practices like, tillage and tilling, resulting in the shortening of the *P. sojae* infection period. Moreover, oospores can also be buried deeper in the soil profile as a result of tillage (Grau et al. 2004). The primary inoculum of *P. sojae* oospore can survive in the soil for many years, therefore, soybean-corn rotation for managing damping off caused by *P. sojae* may not be an effective option (Yang 1997). Nevertheless, planting of resistant soybean cultivar in soybean-corn rotation showed less stand and yield loss compared to soybean mono-cropping (Schmitthenner and Williams 1962). The effect of five years of monoculturing with susceptible, tolerant and resistant cultivars have been demonstrated by Anderson (1986) and they found severe disease in the sixth year on plots previously planted with susceptible and tolerant cultivars while, moderate disease was observed on planted previously with resistant cultivars. The difference in the disease development in the study by Anderson (1986) can be explained by the fact that more oospores are formed more on susceptible and tolerant cultivars than in resistant ones (Anderson 1986; Hartman et al. 1999).

Soybean-*P. sojae* pathosystem

In soybean-*P. sojae* pathosystem the interaction between the pathogen and host follows the gene for gene concept proposed by H. H. Flor (Flor 1971), which assumes that for each *Rps* gene for resistance in the host there is a corresponding avirulence gene in the pathogen. The interaction between a gene for resistance in the host and a gene for avirulence in pathogen results in the resistance reaction in the host known as incompatibility consequently causing in induced resistance. Induced resistance can be defined as the activation of defense mechanisms in host in response to the infection by the causal pathogen (Misaghi et al. 1982). Avirulence gene in the pathogen codes for an elicitor

that that directly or indirectly interacts with the product of the corresponding gene for resistance (Parker et al. 2009). However, susceptibility or compatibility on the other hand is the response (passive or non-induced) that comes due to the absence of avirulence gene in the pathogen and/or absence of resistance gene in the host (Misaghi et al. 1982).

The genetic basis of the host specificity exhibited by physiological races or pathotypes in the *P. sojae*-soybean system can be explained by this concept. Pathogen diversity in *P. sojae* has been assessed traditionally through a virulence test using a bean soydifferential set. There are several soybean lines (7 to 14 soybean lines) each of which contains one resistance gene (*Rps*) to *P. sojae* and a universal susceptible (Williams) are used to characterize *P. sojae* races or pathotypes (Dorrance et al. 2004; Flor 1971). Based on the compatible (susceptible) and incompatible (resistant) reactions on differential lines more than 55 races of *P. sojae* have been described (Dorrance et al. 2003, 2004). Depending on the previously described virulence formula (Herrmann et al. 1999) a race number was given to a pathotype of *P. sojae.* As new virulence gene combinations or pathotypes were continuously emerging in the pathogen the previously described race classification system become complicated (Dorrance et al. 2005). Presently, pathotypes or virulence formulas are used to define virulence patterns based on reactions on a differential. The pathotype system can generate more information as pathotype specifies which *Rps* genes are compatible with the isolate (Robertson et al. 2009). There are more than 200 known pathotypes of *P. sojae* that are already defined (Dorrance et al. 2003), which implies that this pathogen population has high genetic variation in virulence in nature.

From the early surveys in the United States, it was found that virulence to multiple resistance genes was already common in some regions (Schmitthenner et al. 1994; Tooley

et al. 1984; Xue et al. 2015). More recent surveys on *P. sojae* pathotype population suggested that the pathoype populations are adapting to deployment of *Rps* resistant genes (Anderson et al. 2012; Dorrance et al. 2003; Kaitany et al. 2001; Nelson et al. 2008). Nelson et al. (2008) recovered 157 *P. sojae* isolates from 5 to 20 counties and noticed that *Rps*1a is the most commonly defeated *Rps* gene among the 157 isolates. During 2012 to 2013, 11 states of United States including South Dakota were evaluated for pathotype diversity of *P. sojae*. Across all 11 states (Iowa, Indiana, Illinois,Kansas, Michigan, Minnesota, Missouri, Nebraska, New York, Ohio and South Dakota), 36 to 100% of the collected isolates were virulent toward *Rps*1a, *Rps*1b, *Rps*1c,and *Rps*1k, whereas virulence of *P. sojae* isolates against *Rps*6 and *Rps*8 was found to be less than 36 and 10% respectively (Dorrance et al. 2016). Recently, Stewart et al. (2016) studied the population genetic structure by using one isolate from each of 17 fields (2008 to 2010), 33 fields (1997 to 2010), and 20 fields (2002 to 2004) in Iowa, Ohio, and South Dakota, respectively, as well as multiple isolates from individual fields in Iowa, Ohio, and Missouri. Stewart et al. (2016) found that differentials with *Rps*1a, *Rps*1c, *Rps*1k and *Rps*3a genes were most commonly defeated by the *P. sojae* isolates recovered from 2002 to 2004 in South Dakota.

Interaction of *P. sojae* with the soybean cyst nematode (SCN)

Ecologists define interaction as a relationship between two or more organisms that affects the growth, survival or reproduction of the participants. While nematodes are quite capable of causing severe plant injury and reduction in crop production, they are often involved with other disease causing organisms occupying the same ecological niche. Such associations leading to more than additive damage are referred as “complex diseases”, which means the presence of two or more disease causing organisms (Jenkins 1964). As

for interaction between SCN and *P. sojae*, an additive interaction was observed in a study by Adeniji et al. (1975). The lesion length caused by *P. sojae* race 1 (showing virulent reaction on soybean differential with *Rps*7 gene) was higher on a susceptible soybean cultivar ('Corsoy') in the presence of SCN race 3 (*H. glycines* (HG) Type 0) when compared to the lesion length caused by *P. sojae* by itself on 'Corsoy' (Adeniji et al. 1975). In a study by Kaitany et al. (2000), the incidence of *P. sojae* at high and low fumigated SCN condition was assessed and it was observed that *P. sojae* incidence can increase on soybean plants stressed from SCN infestation.

Project and research justification

In South Dakota, a study on Phytopthora root and stem rot of soybean conducted by Draper and Chase (2001) showed that race 1, race 3, race 4 and race 25 of *P. sojae* were most common in South Dakota. A more recent survey in South Dakota conducted by Stewart et al. (2016) showed that soybean differentials with *Rps*1a, *Rps* 1b, *Rps*1c, *Rps*1k and *Rps*3a genes were mostly overcome by the isolates of *P. sojae*. Therefore it is evident that the complexity of *P sojae* race/pathotypes may be increasing over time, especially in the last 15 years. However, the information on the current status on the pathotype diversity of *P. sojae* in South Dakota is limited and the available information is not sufficient for soybean farmers to make informed decisions when selecting cultivars with tolerance or resistance to *P. sojae* for use in their fields.

Combination of major gene resistance with other management strategies can help in managing yield losses occurring from a specific crop disease. One of the options might be to pyramid several major resistance genes into a single cultivar with the hope that the pathogen will not be able to undergo a sequence of mutations corresponding to each

resistance gene. A second option is to generate disruptive selection by rotating major gene resistance through time and space or by growing mixtures of cultivars with different resistance genes inserted into the cultivar. A third option would be the use of partial resistance. However, only a few commercial cultivars with high levels of partial resistance are currently available, mainly due to challenges faced by breeders in incorporating partial resistance into the desired germplasm. For *P. sojae*, since the complexity of the virulence pathotypes continues to increase in soybean production fields in South Dakota, finding additional sources of resistance and incorporation of this resistance into commercial cultivars in combination with the race specific resistance is necessary to manage Phytophthora root rot effectively. Soybean varieties developed for North America have a very narrow genetic basis, which makes the crop especially susceptible to abiotic and biotic stress factors. Therefore strategies could be made to identify unexploited resistance sources from wild soybean *Glycine soja* and introduce them into local varieties to enhance their resistance to *P. sojae.* For the evaluation of partial resistance to *P. sojae* inoculum layer test, tray test is more commonly used. However, a more recent, rice grain inoculation method originally developed by Holmes and Benson (1994) was also used for the assessment partial resistance to *P. sojae.* Although the inoculation methods are available for screening of partial resistance in the greenhouse, the qualitative comparison of the three methods have not been performed so far.

Based on a survey of 200 commercial soybean fields in 2014, a few fields identified where SCN and *P. sojae* are known to co-exist (F. Mathew, *unpublished*). In these commercial fields, it is not unlikely that presence of both the pathogens may cause more yield losses relative to the losses from the pathogens by themselves which the farmers may

not be aware of. Characterization of the pathotype diversity of *P. sojae* in the commercial soybean fields in South Dakota have been performed by R. Chowdhury and E. Byamukama (*unpublished*) and pathotypes virulent on all 13 soybean differentials were identified. Therefore, we hypothesize that such pathotypes that are virulent on all 13 soybean differentials can not only affect lesion development on soybean plants, but disease severity caused by *P. sojae* may be enhanced in the presence of SCN.

Therefore our main objectives in this project were:

1) To determine the pathotype diversity of *Phytophthora sojae* infecting soybean in commercial fields in South Dakota
2) To compare inoculation methods and evaluate of partial resistance to *Phytophthora sojae* in a recombinant inbred line (RIL) population derived from the cross between cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916)
3) To examine the interaction between *Phytophthora sojae* and the Soybean Cyst Nematode and on soybean

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CHAPTER 2

Title: Pathotype diversity of *Phytophthora sojae* infecting soybean in commercial fields in South Dakota

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Keywords: *Phytophthora*, pathotype, Soybean

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Abstract

Chowdhury, R. N., Mathew, F., and Byamukama, E. 201X. Pathotype diversity of *Phytophthora sojae* infecting soybean in commercial fields in South Dakota. Plant Dis. XX: 000-000.

Phytophthora root and stem rot is an important disease of soybean (*Glycine max* L.) in South Dakota. Given *P. sojae* pathotype is highly diverse, resistance genes deployed in commercial soybean varieties fail to manage the disease. Therefore, this study was initiated to determine the pathotype diversity of *P. sojae* in South Dakota. A total of 114 *P. sojae* isolates were soil baited from a total of 384 soybean fields in South Dakota from 2013 to 2015. A total of 70 isolates were pathotyped using the hypocotyl inoculation technique with 13 soybean differentials. Of the 70 *P. sojae* isolates, 50 pathotypes were identified and the pathotypes ranged from being virulent on one *Rps* gene (*Rps*7), to being virulent

on all 13 *Rps* genes. We found 96, 93, 87, 84, 84 and 79% of the isolates were virulent on differentials carrying *Rps*7, *Rps*1a, *Rps*1k, *Rps*1b, *Rps*1c, and *Rps*1d genes. The mean complexity ranged from 6.58 to 6.90 and the Shannon Diversity index ranged from 2.4 to 2.76 for the three years. Our result suggests that *P. sojae* population in South Dakota is diverse and use of partially resistant soybean cultivars by farmers should be combined with other disease management strategies.

Introduction

Phytophthora root and stem rot of soybean (*Glycine max* L) is caused by the pathogen, *Phytophthora sojae*, Kaufmann and Gerdemann (syn. *Phytophthora megasperma* f. sp. *glycinea* Kuan and Erwin). *Phytophthora sojae* belongs to the division Oomycota and genus *Phytophthora*. The pathogen is known to infect soybean plants at all growth stages throughout the growing season. For instance, typical pre- and post-emergence damping-off can develop in the soybean seedlings, while root rot and stem lesions on soybean plants develop in the later or reproduction growth stages of soybean (Schmitthenner 1985). Many soybean-producing countries like, Argentina, Canada, China, Japan, and the United States have reported soybean yield losses from Phytophthora root and stem rot (Dorrance and Grunwald 2009).

In the United States, Phytophthora root and stem rot ranked third among diseases that most suppressed soybean yield from 1996 to 2007 (Wrather and Koening 2009). The disease caused an approximate loss of $338 million (93 thousand metric ton) of revenue to producers according to the 2014 market values for soybean (Bradley et al. 2014). In South

Dakota, Phytophthora root and stem rot is currently one of the most yield-limiting soybean diseases in South Dakota and associated statewide losses are between 4% and 6% each year (Draper and Chase 2001).

Phytophthora sojae overwinters as oospores in crop residue or soil which serves as the primary inoculum. Under suitable moisture and temperature conditions, the dormancy of oospores is broken and produce sporangia. When soil is flooded, sporangia release zoospores which are attracted to root exudates released by the soybean plants (Morris et al. 1998). The zoospores encyst on the root surface and produce a germ tube that grows into the host tissue (Schmitthenner 1985). At the end of the germ tube, *P. sojae* forms an appressorium to penetrate into the host tissue. Haustoria are produced by *P. sojae* to uptake nutrients and colonize the plant (Schmitthenner 1985). Infected soybean plants will experience wilting and chlorosis over time, eventually leading to plant death.

The most effective way to manage Phytophthora root and stem rot of soybean has been through the use of resistant cultivars with single resistance genes (*Rps*). The effectiveness of these genes has been lost progressively as new races/pathotypes of the pathogen have appeared. Recently, Stewart et al. (2016) studied the population genetic structure by using one isolate from each of 17 fields (2008 to 2010), 33 fields (1997 to 2010), and 20 fields (2002 to 2004) in Iowa, Ohio, and South Dakota, respectively, as well as multiple isolates from individual fields in Iowa, Ohio, and Missouri. For almost all of the populations (except three with low population size), a high level of pathotype diversity and a low to moderate level of genotypic diversity was found among the populations for both between states and within field variation. For example, the *P. sojae* isolates collected

in Ohio had greater virulence complexity and pathotype diversity than South Dakota and Iowa (Stewart et al. 2016).

Pathotype diversity in *P. sojae* has been assessed traditionally based on reaction of sets of 7 to 13 soybean differentials, each of which contains one resistance gene (*Rps*) to *P. sojae* that are used to characterize *P. sojae* races or pathotypes (Dorrance et al. 2004; Flor 1971). More than 55 described races of *P. sojae* have been identified on the basis of compatible (susceptible) and incompatible (resistant) reactions on differential lines (Dorrance et al. 2003, 2004). A race number was given to a pathotype of *P. sojae* with a previously described virulence formula depending on which resistance genes in the soybean differentials were overcome. As new virulence gene combinations or pathotypes were continuously emerging in the pathogen the old race classification system became complex and is no longer used (Dorrance et al. 2005). Presently, pathotypes and octal codes are used to describe virulence patterns based on reactions on a differential and the pathotype can be more informative since it indicates which *Rps* genes are compatible with the isolate (Robertson et al. 2009). There are now more than 200 known pathotypes of this pathogen (Dorrance et al. 2003) which suggests that *P. sojae* is a highly diverse pathogen.

Surveys in few of the soybean producing states in the United States suggest the *P. sojae* pathotype population is changing over time. For example, *P. sojae* race 7 was the most prevalent race in Ohio followed by race 9 and race 3 between 1978 and 1980 (Schmitthenner et al. 1994). The subsequent areas were surveyed again after 10 years and it was found that *P. sojae* races 1, 3, 4, 7 and 8 were most prevalent. Between 1997 and 1999, 34 additional pathotypes were reported and predominant races were race 1 and race 25 followed by races 3 and 4 (Dorrance et al. 2003). In Iowa, the survey results from 1966

to 1973 showed the presence of only race 1 (Tachibana et al. 1975) in the soybean fields and from 1991 to 1994 race 3 was predominant (Yang et al. 1996), but the survey from 2001 to 2002 showed that race 3 was replaced by races 25 and 35 (Niu 2004). Several similar findings have been reported in Illinois (Lavallette et al. 1981), Indiana (Abney et al. 1997) and Michigan (Kaitany et al. 2001). In South Dakota, a study was conducted by Draper and Chase (2001) on Phytopthora root and stem rot and they found that race 1, race 3, race 4 and race 25 was most common. Recent survey conducted by Stewart et al. (2016) with 20 *P. sojae isolates* from 2002 to 2004, showed that soybean differentials having *Rps*1a, *Rps*1c, *Rps*1k and *Rps*3a genes were mostly overcome by *P. sojae* isolates in South Dakota. It is evident from these data that the complexity of *P sojae* race/pathotypes has been increasing over time. However, information on the current status on the pathotype structure of *P. sojae* from several counties and fields is not sufficient for soybean farmers to make informed decisions when selecting soybean cultivars with tolerance or resistance to *P. sojae*. The objective of this study was to determine the pathotype diversity of *P. sojae* causing Phytophthora root and stem rot of soybean in commercial fields in South Dakota.

Materials and methods

Survey, soil baiting and isolation of *P. sojae*

Soil samples were collected from a total of 384 soybean fields in South Dakota between 2013 and 2015 (Table 4.1). In 2013, soil samples were collected from 216 fields and 28 counties. In 2014, soil samples were collected from a total of 37 fields covering 8 counties. In 2015, soil samples were collected from a total of 131 fields in 27 counties. Soybean fields were sampled at every 8 km or until a soybean field was located in each county. In each soybean field, approximately 7570.82 ml of soil were collected from the

upper 15 cm of the top soil layer from at least 3 random locations in the field and at least 30 m away from the edge.

To recover *P. sojae* isolates from the soil samples, a soil baiting method was used (Dorrance et al. 2008). Briefly, the soil samples were mixed well and transferred into styrofoam cups (473 ml, Draft container corporation, Mason, MI) with three replications. The cups were then flooded for 24 h using tap water, drained and air dried until the moisture content approached approximately –300 mb matric potential (the wet soil cracks or pulls away from the side of container when the moisture content approached -300 mb matric potential). The cups containing the soil were placed in polyethylene bags and incubated at room temperature for a total of 2 weeks. Following the incubation period, five seeds of the susceptible soybean cv. Williams (provided by Dr. Anne E. Dorrance, The Ohio State University, Columbus, OH) were placed on top of the soil in the cups and covered with wet coarse vermiculite. Three days after planting, when the seedling roots were 5 cm long, the cups were flooded again for 24 h and were placed on greenhouse benches to drain the water. Ten days after planting, seedlings were removed from the Styrofoam cups and each seedling was washed under tap water for 30 min, then washed with antimicrobial soap (Equate^{R}, Bentonville, AR) in order to remove soil off the plants (Dorrance et al. 2008). After soil was removed, roots were kept under running tap water for 30 min. Following that, soybean roots were disinfested with 0.05% NaOCl for 30 s, washed in sterile distilled water and dried on a sterile paper towel. Small pieces of the root (approximately 1 cm) were excised aseptically around the soil line and placed on the selective medium PBNIC (Schmitthenner and Bhat 1994) with some modifications [40 ml V-8 juice (Campbell's, Camden, NJ); 0.6 g $CaCO_3$; 0.2 g Bacto yeast extract (Becton, Dickinson and Company,

Erembodegem, Belgium); 1.0 g sucrose (Sigma-aldrich, St Louis, MO); 20.0 g agar (Sigma-Aldrich, St Louis, MO) in 1000 ml distilled water]. The entire disc of agar medium was inverted in the petri plate, covering soybean root pieces in order to limit the bacterial growth. The PBNIC plates containing mycelial plugs of *P. sojae* were incubated for five days at 25°C in dark.

Phytophthora sojae cultures growing on the PBNIC medium were characterized by the slow growth of dense white mycelium with right-angle branching of coenocytic hyphae (Jackson et al. 2004). After that, mycelial plugs were removed from the leading edges of colonies and transferred to petri plates containing lima bean agar (100 ml lima bean broth and 20 g agar in 1000 ml distilled water). After 2 to 3 days of incubation at 22ºC and in dark, all the colonies were examined with a microscope (at 40X magnification) for characteristic appearance of mycelium and for oospore formation. Oospores were formed on LBA within 3 to 4 days. In order to get pure *P. sojae* isolates, fungal colonies were hyphal-tipped and transferred to PBNIC plates for the second time and the procedure of inverting the PBNIC plates and transferring the mycelial plugs to LBA (lima bean agar) plates was repeated as described above. After 3 to 5 days, mycelial plugs were removed from the leading edges of colonies and transferred to potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ). Isolates were stored in freezer (at 15ºC) until their inoculation on to the 13 differentials. All isolates were confirmed as *P. sojae* by growing them on full strength PDA under dark at 25ºC, since the pathogen does not grow on full strength PDA (Kaufmann et al. 1958).

Molecular verification of the recovered *P. sojae* isolates were done by amplifying approximately 850 bp of the ITS region of randomly selected 20 *P. sojae* isolates (28%).

Fungal DNA for each of the 20 *P. sojae* isolates was extracted from the mycelia grown on diluted V8-juice broth with a Wizard Genomic DNA Purification Kit (Promega Inc., Madison, WI) and the internal transcribed spacer (ITS) gene region was amplified using ITS4 and ITS6 primers (Grünwald et al. 2011). The PCR amplicons were sent for sequencing for DNA sequencing (Functional Biosciences Inc., Madison, WI). The ITS sequences of the 20 *P. sojae* isolates was analysed using Basic Local Alignment Search Tool nucleotide (BLASTN) at GenBank nucleotide database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Pathotype characterization of *P. sojae* isolates

Of the 114 *P. sojae* isolates recovered from soil samples collected from commercial soybean fields in three different years in South Dakota, 70 *P. sojae isolates* were randomly selected (19 fields of 16 counties in 2013, 20 fields from 9 counties in 2014 and 31 fields from 17 counties in 2015) for pathotype characterization. To pathotype the *P. sojae* isolates that were recovered from the soil samples from 2013 to 2015, the hypocotyl inoculation technique (Dorrance et al. 2008) was adopted. Fifteen seeds of 13 differential soybean lines were sown in each styrofoam cup (473 ml, Draft container corporation, Mason, MI) and grown for 7 days at 25 to 28^{o}C under 16 h photoperiod with a light intensity of 1000 $\mu Em^{-2}s^{-1}$ and watered daily. The 13 differentials used in this study were obtained from the USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC and these included Harlon (*Rps*1a), Harosoy 13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps* 1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2*)*, L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8). Soybean cv. Williams was used as the susceptible

check in this study (Dorrance et al. 2004). A total of 30 plants (10 plants with three replications) of each differential were inoculated with each of the 70 *P. sojae* isolates. The experiment was set up as a completely randomized design with three replications (styrofoam cups) per treatment (*P. sojae* isolate) and was repeated once.

To inoculate the differentials for pathotyping *P. sojae* isolates, a slurry was prepared by cutting the 15-day old LBA culture of *P. sojae* in strips and placing them in a 10-ml syringe (Dorrance et al. 2008). The agar culture strips were forced through the syringe twice. Using a 18-gauge needle, a slit (approximately 1 cm long) was made below the cotyledons on the hypocotyl of seven day old seedlings of each of the 15 differentials. About 0.2 to 0.4 ml (approximately 200 to 400 cfu/ml) of the culture slurry was placed into the slit of the seedlings with the syringe. After inoculation, the plants were incubated in a dew chamber (95% humidity) for the next 24 h, at a temperature range of 20 to 22°C in the dark. After 24 h of incubation, the soybean plants were placed in a greenhouse at temperatures ranging from 22 to 28°C under natural light. At 5 to 7 days after inoculation, pathogenicity of each isolates was evaluated. Plants that developed brown expanding lesions on the stem were classified as susceptible, while plants that developed a hypersensitive reaction defined by "a slight necrotic lesion around the wound where inoculation was performed" were classified as resistant (Dorrance et al. 2008). The differential was considered susceptible when at least 7 of the 10 seedlings developed an expanding necrotic brown lesion. A differential was considered resistant if 70% or more of the plant inoculated with *P. sojae* survived (Dorrance et al. 2008).

To determine the pathotypes of *P. sojae* isolates, the reverse octal format previously described for *P. sojae* (Dorrance et al. 2003), *Rhyncosporium secalis*

(Oudemans) Davis (Goodwin et al. 1990) and for *Phytophthora infestans* (Montagne) de Bary (Goodwin et al. 1995) was adopted. As per as the reverse octal format, the differentials are organized in three groups and each group of three differentials is coded as one octal digit. Based on the susceptible or resistant responses of each differential within a set the octal numbers were assigned for each pathotype; 0 indicate a resistant reaction after inoculation and 1 indicate a susceptible reaction. The intermediate ratings were not considered. The soybean differentials for *P. sojae* were grouped into octal digits as follows: The first octal digit contained *Rps*1a, *Rps*1b, *Rps*1c; the second octal digit contained *Rps*1k, *Rps*2, and *Rps*3a, the third octal digit contained *Rps*3b, *Rps*3c, and *Rps*4; and the fourth octal digit contained *Rps*6, *Rps*7, and *Rps*8. Octal digits were assigned numbers as follows: 000 = 0; 100 = 1; 010 = 2; 110 = 3; 001 = 4; 101 = 5; 011 = 6; and 111 = 7. For the complete set of isolates, simple diversity (which measures the proportion of distinct pathotypes as compared to the number of isolates evaluated), Shannon diversity (that indicates the evenness of distribution of virulence phenotypes within a sample), Gleason diversity (which indicates phenotypic richness) and mean complexity indices (that represents the number of *Rps* genes with which an isolate has a susceptible interaction) were calculated using the HaGiS spreadsheet program (Hermann et al. 1999). In addition, the mean complexity (mean number of differential that had susceptible reaction following inoculation) of the *P. sojae* isolates was also calculated.

Results

Survey, soil baiting and isolation of *P. sojae*

From a total of 384 fields across three years, 114 isolates of *P. sojae* were recovered and 70 isolates of *P. sojae* (19 from 2013, 20 from 2014 and 31 from 2015) from a total of

67 fields were evaluated for pathotype characterization on 13 differential cultivars (Table 2.4). The identity of *P. sojae* isolates were confirmed by matching the ITS sequence of the isolates of *P. sojae* in this study with that of the *Phytophthora sojae* isolate SDSO_9-72 (Accession # KU211500.1) with identities = 834/834 (100%) and gaps = 0/ 834 (0%).

Pathotype characterization of *P. sojae* isolates

All the isolates caused disease on Williams (universal susceptible) and none of the *Rps* gene differentials conferred resistance to all isolates of *P. sojae* in this study. Among the 70 isolates evaluated, 50 pathotypes were identified and the pathotypes ranged from being virulent on one *Rps* gene represented by virulence formula 00001 (formally race 1), to being virulent on all 13 *Rps* genes represented by virulence 77771 (Table 4.2). Pathotypes with phenotype 1a, 1b, 1c, 1d, 1k, 7 was the most common, covering 36% of the total isolates followed by pathotype 1a, 1b, 1c, 1d, 7 comprising 14% of the total isolates (Table 2.3).

In 2013, a total of 59 *P. sojae* isolates were recovered from 216 soybean fields (Table 4.1) and among the 59 *P. sojae* isolates, 19 *P. sojae* isolates were randomly selected and used for pathotype characterization (Table 2.2). Among the 19 *P. sojae* isolates, 100% were virulent on *Rps*1a, *Rps*1c and *Rps*7, while 84% of the *P. sojae* isolates were virulent on *Rps*1b, *Rps*1d and *Rps*1k. However, none of the *P. sojae* isolates were able to produce disease on *Rps*2. Of the *P. sojae* isolates collected, 26% were virulent on *Rps*3c and *Rps*6. About 16% of the *P. sojae* isolates were virulent on *Rps*3a, *Rps*4 and *Rps*5 while 5% were virulent on *Rps*3b (Fig. 2.1).

In 2014, a total of 21 *P. sojae* isolates were recovered from 37 soybean fields (Table 1.1) and among the 21 *P. sojae* isolates, 20 *P. sojae* isolates were randomly selected and

used for pathotype characterization (Table 2.2). Among the 20 *P. sojae* isolates, more than 80% of the *P. sojae* isolates were virulent on *Rps*1a, *Rps*1d and *Rps*7, while 60% of the *P. sojae* isolates were virulent on *Rps*1b, *Rps*1c and *Rps*1k. Of the *P. sojae* isolates collected, 30% were virulent on *Rps*2, *Rps*3a and *Rps*4. About 25% of the *P. sojae* isolates were virulent on *Rps*6 (Fig. 2.2).

In 2015, a total of 34 *P. sojae* isolates were recovered from 131 soybean fields (Table 2.1) and among the 34 *P. sojae* isolates, 31 *P. sojae* isolates were randomly selected and used for pathotype characterization (Table 2.2). Among the 31 *P. sojae* isolates, 95% were virulent on *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k and *Rps*7, while 71% of the *P. sojae* isolates were virulent on *Rps*1d. Of the *P. sojae* isolates collected, 23% were virulent on *Rps*3c and *Rps*3c,19% of the *P. sojae* isolates were virulent on *Rps*3b, *Rps*4 and *Rps*6 while 10% were virulent on *Rps*2 (Fig. 2.3).

As for the diversity indices that were estimated from our data set, simple diversity was greatest in 2014 (0.85) followed by 2013 (0.74). However, simple diversity was low in 2015 (0.55) (Table 2.4). Gleason's index was greatest in 2014 (5.34) followed by 2015 (4.66) and 2013 (4.42) (Table 2.4). Shannon's index was higher in 2014 (2.76) as compared to 2013 (2.45) and 2015 (2.40) (Table 2.4). The mean complexity of these isolates recovered from the field across the year ranged from 6.58 (2013) to 6.90 (2015) (Table 2.4).

Discussion

In our survey, a total of 114 isolates of *P. sojae* were recovered from soil samples collected from 384 fields covering 30 counties in South Dakota during 2013 and 2015. Among the 114 isolates of *P. sojae* that were recovered from 384 fields, 70 isolates were

randomly selected and used for pathotype evaluation. Pathotypes with phenotype *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, and *Rps*7 were the most common, which covered 36% of the total 114 isolates. This was followed by pathotype *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, and *Rps*7, which compromised 14% of the total isolates. In 2013, over 80% of the *P. sojae* isolates pathotyped were virulent on *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k and *Rps*7 genes, while more than 25% of the *P. sojae isolates* were virulent on *Rps*3c and *Rps*6 genes. Less than 20% of the *P. sojae* isolates were virulent on *Rps*3a, *Rps*4 and *Rps*5 genes, 5% isolates virulent on *Rps*3b gene and none of the isolates were virulent on *Rps*2. In 2014, more than 65% of the *P. sojae* isolates were virulent on *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k and *Rps*7 while more than 25% of the *P. sojae* isolates were virulent on *Rps*2, *Rps*3a, *Rps*3b, *Rps*3c, *Rps*4, *Rps*5, and *Rps*6 genes. In 2015, over 90% of the *P. sojae* isolates showed susceptible reaction on *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k and *Rps*7 genes. More than 23% of the *P. sojae* isolates were virulent on *Rps*3a, *Rps*3c and *Rps*5 genes and more than 10% of the *P. sojae* isolates were able to produce disease on *Rps*2, *Rps*3b, *Rps*4 and *Rps*6 genes. For the three years, the mean complexity ranged from 6.58 to 6.90 and the Shannon Diversity index ranged from 2.40 to 2.76.

In South Dakota cultivars containing *Rps*1c and *Rps*1k (69%) are commonly grown (E. Byamukama, *personal communication*) and our overall results suggest that the *P. sojae* populations in South Dakota may have adapted to the commonly used resistance genes (*Rps*1a, *Rps*1c and *Rps*1k) in soybean cultivars (Dorrance et al. 2003). For example, 84% of the *P. sojae* isolates that were collected in this study defeated *Rps*1c gene (Fig. 2.2) and those isolates were recovered from 61 of the 70 fields where *P. sojae* was detected (Table 2.3). Similarly, 75% of the *P. sojae* isolates that were pathotyped in this study defeated the

*Rps*1k gene and these pathotypes were recovered from 61 of the 70 fields (87%). This may be the result of repeatedly using the resistant cultivars with *Rps*1a, *Rps*1c and *Rps*1k over time which rather imposes a selection pressure to the pathogen enabling the breakdown of the resistance to these *Rps* genes. For *P. sojae*, previous studies have supported that specific resistance to the pathogen in commercial soybean varieties was overcome due to the selection pressure. For example, a field survey in Ohio by Dorrance et al. (2003) (57 fields 1990 to 1991 and 29 fields in 1997 to 1999) concluded that 96, 65, 73, 78, 51, and 52% of the locations had at least one isolate that were virulent on differentials carry *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a, and *Rps*6, respectively, where the most commonly deployed *Rps* genes for *P. sojae* in commercial soybean cultivars in these locations in Ohio have been *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a and *Rps*6 (Dorrance et al. 2003). In Michigan, Kaitany et al. (2001) showed that 12 and 13% of the isolates were virulent on differentials carrying *Rps*1a and *Rps*7 when *Rps* genes 1a, 1c, 1k, 3, 6 and 7 are deployed in the commercial soybean cultivars either singly or in combinations. In this study, we found 23% of the collected *P. sojae* isolates were able to cause disease on plants with *Rps*3a and *Rps*6 genes which were found in only 16 soybean fields within the three years. Thus, resistance genes *Rps*3a and *Rps*6, which are not as widely used compared to *Rps*1k, may be useful genes to deploy in South Dakota soybean varieties. Moreover, *Rps*3b and *Rps*2 gene which was defeated by 20% and 13% of the total *P. sojae* isolates might also be useful for *Rps* gene deployment in South Dakota.

In our study, we observed that complexity of pathotypes in South Dakota is continuing to increase when compared with results from the previous surveys. For example, recent study on the population structure of *P. sojae* among and within fields in

South Dakota by Stewart et al. (2016) reported that mean complexity was 4.6 based on 20 *P. sojae* isolates recovered from one plant per field during 2002 to 2004 (Table 2.5). Dorrance et al. (2016) surveyed on the pathotype diversity in eleven different states in the United States including South Dakota. A total of 29 *P. sojae* isolates were recovered by soil baiting from 5 different fields in South Dakota in 2012 and 2013 and they reported that 15% of the *P. sojae* isolates were virulent on at least six *Rps* genes (Table 2.5). However, in our study we found that 37% of the isolates of *P. sojae* were virulent on six to seven *Rps* genes indicating that number of virulent on numerous genes has increased from the previous surveys. Additionally, none of the *P. sojae* isolates were found virulent on *Rps*2 in 2013 while less than 30% of the isolates were found virulent on R*ps*2 for the following years (2014 and 2015). The difference in mean complexity between our survey and those by Stewart et al. (2016) and Dorrance et al. (2016) may be because the South Dakota sample size for pathotype diversity in our study was much larger. In addition, other factors such as differences in soil sample selection strategies, sampling locations, baiting methods, and use of differential cultivars to obtain and determine the pathotypes of isolates may affect the pathotype diversity over time. However, our study also indicates that the increased complexity as well as new virulence combination in *P. sojae* in South Dakota may likely to be an effect of outcrossing within a field or between fields in the state as hypothesized by Stewart et al. (2016). Stewart et al. (2016) used a total of 21 polymorphic SSR markers for measuring the genotypic diversity of the *P. sojae* isolates collected form the fields of South Dakota and found few number of isolates that share common Multi Locus Group (defined by Stewart et al. (2016) for each *P. sojae* isolate as number and frequency of the alleles at each of the polymorphic marker loci)

Our study has also demonstrated that pathotype variability in *P. sojae* may vary from county to county in South Dakota. For example, none of the isolates of *P. sojae* were being able to overcome the *Rps* genes 2, 3a, 3b, 3c, 4, 5, and 6 between 2013-2015 in Roberts County which is located in the north-eastern part of South Dakota (Figure 2.4). While in Brookings and Turner county that are located in the central-eastern and south-eastern part of South Dakota respectively, the *P. sojae* isolates were able to defeat all of the *Rps* genes with varying frequency during the three years (2013-2015) (Figure 2.5 and Figure 2.6). Local agricultural practices, sample size, and history of deploying *Rps* genes in the locality might be contributing to pathotype variation in the county level. Additionally, the *P .sojae* pathotype among counties may be diverse because of outcrossing caused by the homothallic pathogen, *P. sojae*. In our study, we have only compared pathotype diversity of *P. sojae* between fields, future research should focus on sampling within fields to understand the overall pathotype structure of *P. sojae* in South Dakota.

Relatively, the pathotype variability for *P. sojae* populations in South Dakota is not as diverse as in other states of United States, such as Michigan, Illinois, Indiana and Ohio (Abney et al. 1997; Dorrance et al. 2003; Kaitany et al. 2001; Malvick, et al. 2004; Stewart et al. 2016). For example, 54 and 56 pathotypes were identified in two commercial soybean fields in Ohio by Dorrance et al. (2003). Moreover, study by Robertson et al. (2009) detected 11 and 18 pathotypes from two commercial soybean fields and four different pathotypes from one soil sub samples. However, in our study, we recovered only one unique pathotype from 63% of the fields where we detected *P. sojae*. Although in our study, a single isolate recovered from each field were used for evaluation, sampling of larger number of isolates per field is necessary to understand the possible number of sub

populations and overall pathotype diversity. The Shannon diversity indices which measures the relative differences in pathotype among the *P. sojae* isolates had not changed much over the ten years (Table 2.5). This may be because in South Dakota farmers use corn and soybeans in their crop rotation systems and use of the cultivars with different *Rps* gene in the same field has posed less selection pressure to the *P. sojae* population.

In conclusion, the pathotype diversity of *P. sojae* in South Dakota has increased over time and also the presence of pathotypes that defeat the existing resistant genes have been identified. Of the 50 pathotypes identified, pathotype 1a, 1b, 1c, 1d, 1k was most frequently recovered (36% of the total *P. sojae* isolates). Complexity and virulence of the *P. sojae* population is posed to expand over time, but still the incidence of virulence on a specific *Rps* gene exists only in a proportion of the sampled field (Dorrance et al. 2003; Robertson et al. 2009). Therefore, it might still be recommended to continue with cultivar selection for the management of Phytophthora root and stem rot in commercial fields in order to prevent the yield losses. Based on our study, it might be suggested that *Rps*2, *Rps*3a, *Rps*3b, and *Rps*6 genes may be potential candidate either alone or in combinations for deploying in the commercial soybean cultivar for effective management of *P. sojae* in South Dakota. Deployment of *Rps*3a and *Rps*6 in the commercial soybean cultivars commonly used in South Dakota was suggested by Dorrance et al. (2016), as virulence of *P. sojae* to these genes were detected at low and infrequent incidence upon pathotyping. Moreover, routine survey of existing *P. sojae* pathotypes in commercial soybean production field is also necessary to predict the durability of the *Rps* genes in South Dakota.

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Table 2.1 Year, county information, total number of fields, and number of *P. sojae* isolates recovered from the soil sampled from commercial soybean fields across South Dakota from 2013 to 2015.

Year of Collection	County	Total no of fields	No of fields negative for *P. sojae*	No of *P. sojae* isolates recovered
2013	28	216	157	59
2014	8	37	16	21
2015	27	131	97	34

Table 2.2 Octal code of the most common pathotypes *of Phytophthora sojae* recovered from 67 out of 384 fields sampled in South Dakota during 2013, 2014 and 2015

Year	County[a]	No of fields	Isolate code	Pathotype[b]
2013	Beadle	2	PS-13-BedF5	73441
			PS-13-BedF2	50001
	Bon Homme	2	PS-13-BoHF5	53001
			PS-13-BoHF8	73021
	Brookings	1	PS-13-BroF4	73601
	Davison	1	PS-13-DavF7	73011
	Grant	1	PS-13-Grant	73001
	Hand	1	PS-13-HandF2	73461
	Hutchinson	1	PS-13-HucF3	73101
	Marshall	1	PS-13-MarF5	73001
	McCook	1	PS-13-McCF3	73551
	McPherson	1	PS-13-McPF2	72001
	Miner	1	PS-13-MinF7	73001
	Minnehaha	2	PS-13-MnnF1	73001
			PS-13-MnnF3	73061
	Moody	1	PS-13-ModF5	51001
	Roberts	2	PS-13-RobF4	73001
			PS-13RobF6	70001
	Turner	1	PS-13-TF3	73551

[a] County name where the field is located

[b] The Octal Code was determined with HaGiS, in which 0 indicates an incompatible reaction, and 1 indicates a compatible reaction on the differentials following inoculation. Octal digits were assigned as follows: 000 = 0, 100 = 1, 010 = 2, 001 = 4, 110 = 3, 101 = 5, 011 = 6, and 111 = 7. (Hermann et al. 1999). The first digit is the response to Rps1a, Rps1b, Rps1c, the second digit: *Rps*1d, *Rps* 1k, *Rps*2; the third digit: *Rps*3a, *Rps*3b, *Rps*3c; the fourth digit: *Rps*4, *Rps*5, *Rps*6; and the fifth digit, *Rps*7, *Rps*8.

Table 2.2 (contd.). Octal code of the most common pathotypes *of Phytophthora sojae* recovered from 67 out of 384 fields sampled in South Dakota during 2013, 2014 and 2015

Year	County[a]	No of fields	Isolate code	Pathotype[b]
2014	Brookings	7	PS-14-PF6	73721
			PS-14-PF7	77201
			PS-14-PF4	51001
			PS-14-F8	33001
			PS-14-F8'	77571
			PS-14-F10	77771
			PS-14-BR3	37771
	Clay	3	PS-14-F14	00001
			PS-14-F18	01201
			PS-14-F13	53001
	Codington	1	PS-14-F11	73001
	Grant	1	PS-14-F173	73001
	Lincoln	2	PS-14-F3	01500
			PS-14-F14-6	10000
	Moody	1	PS-14-F137	57071
	Roberts	1	PS-14-RB2	73001
	Turner	2	PS-14-F9	72021
			PS-14-PF10	21021
			E-14-61E	73631

[a] County name where the field is located

[b] The Octal Code was determined with HaGiS, in which 0 indicates an incompatible reaction, and 1 indicates a compatible reaction on the differentials following inoculation. Octal digits were assigned as follows: 000 = 0, 100 = 1, 010 = 2, 001 = 4, 110 = 3, 101 = 5, 011 = 6, and 111 = 7. (Hermann et al. 1999). The first digit is the response to *Rps*1a, *Rps*1b, *Rps*1c, the second digit: *Rps*1d, *Rps* 1k, *Rps*2; the third digit: *Rps*3a, *Rps*3b, *Rps*3c; the fourth digit: *Rps*4, *Rps*5, *Rps*6; and the fifth digit, *Rps*7, *Rps*8.

Table 2.2 (contd.). Octal code of the most common pathotypes *of Phytophthora sojae* recovered from 67 out of 384 fields sampled in South Dakota during 2013, 2014 and 2015

Year	County[a]	No of fields	Isolate code	Pathotype[b]
2015	Brookings	4	PS-15-BroF1	77071
			PS-15-BroF3	73001
			PS-15-BroF4	73001
			PS-15-BBE	73771
	Brown	2	PS-15-BrnF3	73001
			PS-15-F70	72221
	Clark	2	PS-15-F24	33521
			PS-15-CkF5	73001
	Clay	1	PS-15-ClF2	73001
	Codington	2	PS-15-CodF4	73201
			PS-15-CodF8	72001
	Davison	3	PS-15-BE154	73201
			PS-15-DavBE1	73521
			PS-15-DavCT	72201
	Day	1	PS-15-DayF5	02000
	Deuel	3	PS-15-F55	73001
			PS-15-DuF1	73001
			PS-15-DuF2	73021
	Hanson	1	PS-15-F41	72001
	Hamlin	1	PS-15-F53	73001
	Kingsbury	1	PS-15-KinBE	77171
	Miner	1	PS-15-F15	72001
	Moody	2	PS-15-MoF4	73511
			PS-15-MoF3	73001
	Roberts	1	PS-15-RobF1	33001
	Spink	3	PS-15-F23	73441
			PS-15-SpkF4	72001
			PS-15-F71	73571
	Turner	1	PS-15-TF3	77771
	Union	2	PS-15-F30	72001
			PS-15-F32	72001

[a] County name where the field is located

[b] The Octal Code was determined with HaGiS, in which 0 indicates an incompatible reaction, and 1 indicates a compatible reaction on the differentials following inoculation. Octal digits were assigned as follows: 000 = 0, 100 = 1, 010 = 2, 001 = 4, 110 = 3, 101 = 5, 011 = 6, and 111 = 7. (Hermann et al. 1999). The first digit is the response to *Rps*1a, *Rps*1b, *Rps*1c, the second digit: *Rps*1d, *Rps* 1k, *Rps*2; the third digit: *Rps*3a, *Rps*3b, *Rps*3c; the fourth digit: *Rps*4, *Rps*5, *Rps*6; and the fifth digit, *Rps*7, *Rps*8

Table 2.3 Isolates of *Phytophthora sojae* that shows unique virulence formula baited from soybean field soils collected in South Dakota from 2013 to 2015

No of *Rps* genes on which an isolate was virulent	No of isolates	Virulence formula[a]
1	1	1k
1	1	7
1	1	1a
3	1	1d,3a,3c
3	1	1d,3b,7
3	1	1a,1c,7
4	1	1a,1c,1d, 7
4	1	1b,1d,5,7
4	1	1a,1c,1d,7
4	1	1a,1b,1c,7
5	6	1a,1b,1c,1k,7
5	3	1a,1b,1d,1k,7
5	1	1a,1b,1c,1k,7
5	2	1a,1c,1d,1k,7
6	16	1a,1b,1c,1d,1k,7
6	1	1a,1b,1c,1k,3b,7
6	1	1a,1b, 1c,1k,5,7
7	1	1a,1b,1c,1k,3b,5,7
7	2	1a,1b,1c,1d,1k,3b,7
7	2	1a,1b,1c,1d,1k,5,7
7	1	1a,1b,1c,1d,1k,3a,7
7	1	1a,1b,1c,1d,1k,4,7
8	2	1a,1b,1c,1d,1k,3c,6,7
8	1	1a,1b,1d,1k,3a,3c,5,7
8	1	1a,1b,1c, 1d, 1k,2,3b,7
8	1	1a,1b,1c,1d,1k,3b,3c,7
8	1	1a,1b,1c,1d,1k,5,6,7

[a] Pathotype of the isolate was determined by inoculating 13 differentials, which included Harlon(*Rps*1a), Harosoy 13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps*1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible check) (Dorrance et al. 2004).

Table 2.3 (contd.). Isolates of *Phytophthora sojae* that shows unique virulence formula baited from soybean field soils collected in South Dakota from 2013 to 2015

No of *Rps* genes on which an isolate was virulent	No of isolates	Virulence formula[a]
9	2	1a,1b,1c,1d,1k,3c,5,6,7
9	1	1a,1b,1c,1d,1k,3a,3c,4,7
9	1	1a,1b,1c,1d,1k,3a,3c,5,7
9	1	1a,1c,1d,1k,2,4,5,6,7
9	1	1a,1b,1c,1d,1k,3c,5,6,7
10	1	1a,1b,1c,1d,1k,2,4,5,6,7
10	1	1a,1b,1c,1d,1k,3a,3b,3c,5,7
10	1	1a,1b,1c,1d,1k,3b,3c,4,5, 7
10	1	1a,1b,1c,1d,1k,3a,3c,4,6,7
10	1	1a,1b,1c,1d,1k,3a,3c,4,6,7
11	1	1a,1b,1c,1d,1k,3a,3c,4,5,6,7
11	1	1a,1b,1c,1d,1k,2,3a,4,5,6,7
12	1	1a,1b,1c,1d,1k,3a,3b,3c,4,5,6,7
12	1	1a,1b,1c,1d,1k,2,3a,3c,4,5,6,7
12	1	1a,1b,1d,1k,2,3a,3b,3c,4,5,6,7
13	3	1a,1b,1c,1d,1k,2,3a,3b,3c,4,5,6,7

[a]Pathotype of the isolate was determined by inoculating 13 differentials, which included Harlon(*Rps*1a), Harosoy 13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps*1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible check) (Dorrance et al. 2004).

Table 2.4 Comparison of the pathotype diversity indices among isolates of *Phytophthora sojae* collected from individual fields in South Dakota during the year 2013 to 2015.

Year	Number of fields	Number of pathotypes [a]	Indices of diversity[f]			
			Simple diversity[b]	Gleason's index[c]	Shannon diversity index[d]	Mean complexity[e]
2013	19	15	0.74	4.42	2.45	6.58
2014	20	17	0.85	5.34	2.76	6.85
2015	31	18	0.55	4.66	2.40	6.90

[a] Pathotype of the isolate was determined by inoculating 13 differentials, which included Harlon(*Rps*1a), Harosoy 13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps*1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible check) (Dorrance et al. 2004).

[b] Simple diversity was calculated based on the proportion of distinct pathotypes relative to the number of isolates evaluated.

[c] Gleason's index, an indication of phenotypic richness

[d] Mean complexity was calculated based on the average number of *Rps* gene differentials on which each isolate of *P. sojae* can cause disease.

[e] Shannon diversity index was calculated with the HaGiS spreadsheet program (Herrmann et al. 1999).

[f] Diversity indices were calculated using formula presented in Groth and Roelfs (1987) using the spreadsheet program HaGiS (Hermann et al. 1999).

Table 2.5 Comparison of the number of fields sampled, the number of fields where *P. sojae* was recovered, the number of isolates of *P. sojae* that were collected, and pathotypes along with indices of diversity of isolates of *P. sojae* collected in South Dakota between 2013 to 2015 (this study) compared to results obtained in earlier surveys

	Number of [a]				**Indices of diversity[b]**				
Year	Samp	Reco	Iso	Path[c]	Simple	Gleason	Shannon	Mean complexity	Cited
2002 to 2004	20		20	17	…	…	2.76	4.60	Stewart et al. 2016
2012 to 2013	5	5	29	18	0.62	5.05	2.74	…	Dorrance et al. 2016
2013 to 2015	67	70	70	50	0.55	4.66	2.53	6.80	This study

[a] Number of field sampled (Samp), fields from which *P. sojae* was recovered (Reco), Isolates (Iso), and pathotypes (Path).

[b] Diversity indices were calculated using formula presented in Groth and Roelfs (1987) using the spreadsheet program HaGiS (Hermann et al. 1999).

[c]Pathotype of the isolate was determined by inoculating 13 differentials, which included Harlon(*Rps*1a), Harosoy 13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps*1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2), L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible check) (Dorrance et al. 2004).

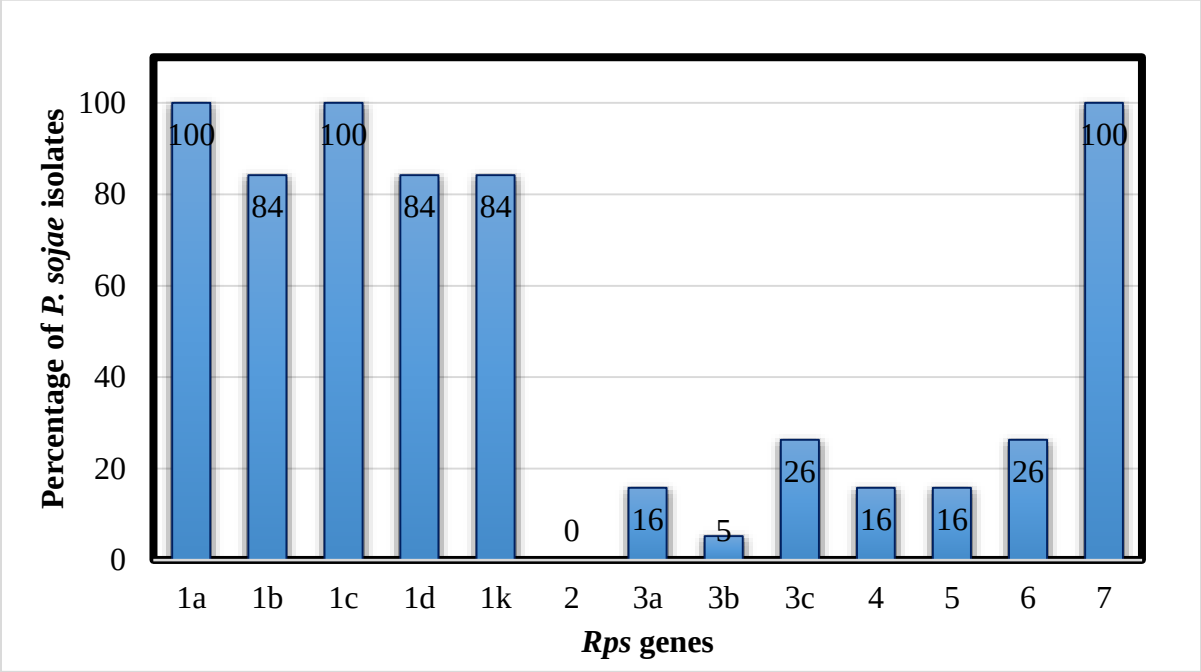

Figure 2.1 Reaction of 19 isolates of *Phytophthora sojae* on thirteen differentials from USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC (OSU). These isolates were recovered from soil samples collected from soybean fields across South Dakota in 2013.

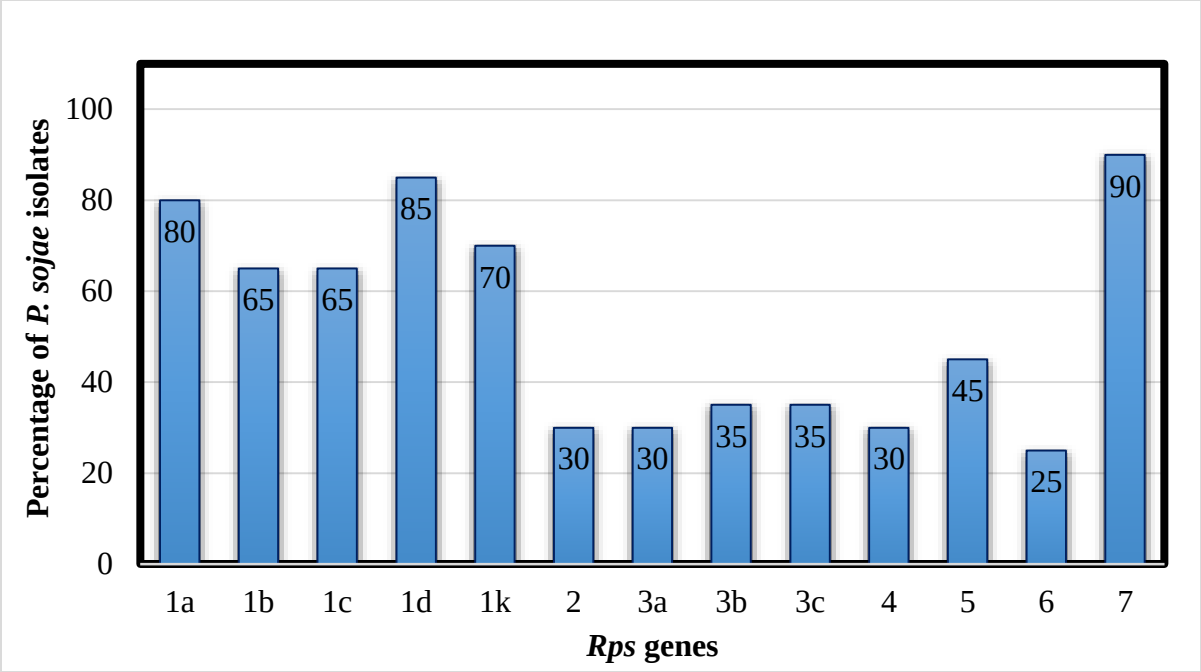

Figure 2.2 Reaction of 20 isolates of *Phytophthora sojae* on thirteen differentials from USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC (OSU). These isolates were recovered isolates of *Phytophthora sojae* recovered from soil samples collected from soybean fields across South Dakota in 2014.

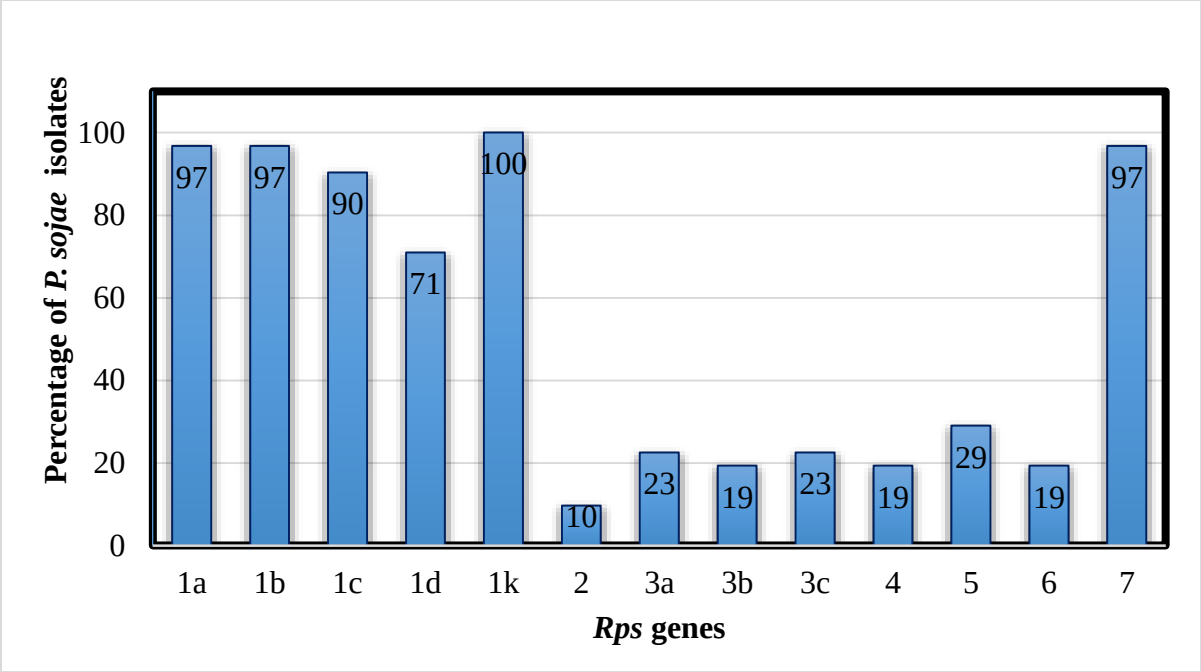

Figure 2.3 Reaction of 31 isolates of *Phytophthora sojae* isolates of *Phytophthora sojae* on thirteen differentials from USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC (OSU). These isolates were recovered from soil samples collected from soybean fields across South Dakota in 2015.

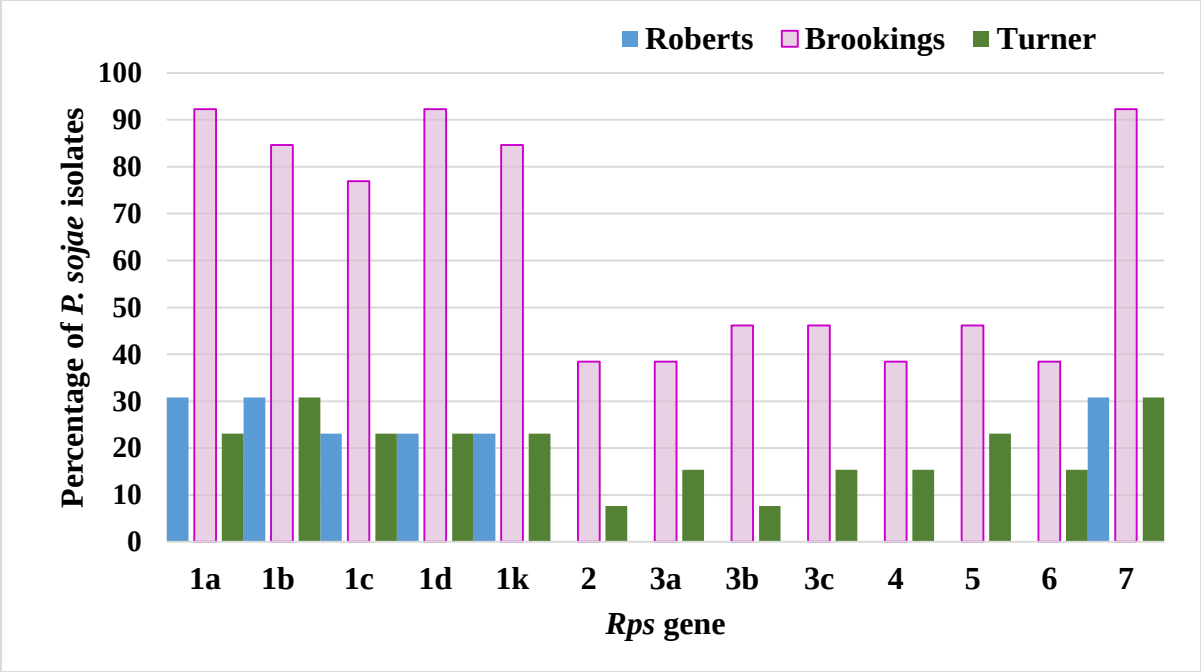

Figure 2.4 Reaction of *Phytophthora sojae* isolates recovered from soil samples collected from soybean fields in Roberts County, Brookings County and Turner County of South Dakota across three years (2013 to 2015) on thirteen differentials from USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC (OSU).

CHAPTER 3

Title: Comparison of inoculation methods and evaluation of partial resistance to *Phytophthora sojae* in a recombinant inbred line (RIL) population derived from the cross between cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916)

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Keywords: partial resistance, *Phytophthora*, inoculation

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Abstract

Chowdhury, R. N., Gu, X., Mathew, F. M. and Byamukama, E. 201X. Comparison of inoculation methods and evaluation of partial resistance to *Phytophthora sojae* in a recombinant inbred line (RIL) population derived from the cross between cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916). Plant Dis. XX: 000-000**.**

To identify a suitable inoculation method to screen soybean genotypes for partial resistance to *Phytophthora sojae,* three methods (inoculum layer test, tray test and rice grain inoculation) were evaluated in the greenhouse. Based on the recovery of *P. sojae* isolate (%) and its correlation with lesion length caused by the *P. sojae* isolates at 7 days after inoculation, the inoculum layer method was selected to screen one hundred recombinant inbred lines (RILs) derived from the cross between *Glycine max* (cv. Surge)

and *Glycine soja* (PI 468916). In order to evaluate partial resistance to two isolates of *P. sojae* [PS-15-TF3 being virulent on 13 differentials and PS-14-F14 being virulent on one (*Rps*7) differential] in the greenhouse, lesion length produced by the pathogen was measured. For PS-15-TF3, 63% of the RILs had significantly shorter lesion length as compared to the moderately resistant parent *Glycine soja*. For PS-14-F14, 39% of the RILs had significantly shorter lesion length as compared to *G. soja.* Upon comparing the response of RILs to both PS-15-TF3 and PS-14-F14, 9 RILs had relatively shorter lesion length than the parents.

Introduction

Genetic host resistance is one of the most effective strategies to manage disease in all cropping systems. On soybean (*Glycine max* L.), two types of genetic host resistance have been reported, partial resistance and single dominant genes (*Rps*) mediated resistance for management of Phytophthora root rot and stem rot of soybean caused by *Phytophthora sojae* Kaufmann and Gerdemann (syn. *Phytophthora megasperma* f. sp. *glycinea* Kuan and Erwin) (Dorrance et al. 2007).

Single dominant genes (*Rps*) mediated resistance confers an immune type of resistance to a limited number of *P. sojae* isolates that carry the cognate avirulence (*Avr*) gene (Gijzen and Qutob 2009). A total of 20 *Rps* loci including 25 alleles have been mapped on soybean genome (Demirbas et al. 2001; Fan et al. 2009; Gao et al. 2005; Lin et al. 2013; Sugimoto et al. 2011; Sun et al. 2011; Wang et al. 2001; Wu et al. 2011; Yao et al. 2010; Zhang et al. 2013; Ping, et al. 2015). Among the described *Rps* genes only seven genes, *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*3a, *Rps*6 and *Rps*8, have been deployed

commercially singly or in combinations. Based on the pathogen inoculum density and environmental condition, *R* gene mediated resistance can be effective for 8 to 15 years (Schmitthenner 1985). However, constantly growing of soybean cultivars with *Rps* genes in North America has subjected *P. sojae* to selection pressure and in the evolution of more virulence pathotypes of the pathogen (Grau et al. 2004). A number of pathotypes of *P. sojae* have been identified that can overcome the resistance conferred by the known *Rps* genes (Dorrance et al. 2003).

In addition to *Rps* gene-mediated resistance, partial resistance is another form of genetic resistance that is used to manage *P. sojae* (Burnham et al. 2003). This type of resistance is controlled by more than one gene and is effective against all physiological races of the pathogen. For example, for *P. sojae*, Jia and Kurle (2008) used 69 plant introduction (PI)s for evaluation of partial resistance to *P. sojae* races 7 and 25 using the inoculum layer method. Among the 69 PIs, 22 PIs had the same level of partial resistance as Conrad to *P. sojae* race 7 while 19 PIs had the same degree of partial resistance to race 25 (Jia and Kurle 2008). Twelve PIs had the same level of partial resistance as Conrad to both *P. sojae* races 7 and 25 (Jia and Kurle 2008). Partial resistance can exert less selection pressure on the pathogen population as they are controlled by polygenes, thus providing more durable and stable resistance (Simons et al. 1970). In South Dakota, based on research by Chowdhury et al. *unpublished* [chapter 1] in which 70 *P. sojae* isolates were evaluated for pathotype diversity, 50 pathotypes were identified and the pathotypes ranged from being virulent on one *Rps* gene represented by virulence formula 00001 (formally race 1), to being virulent on all 13 *Rps* genes represented by virulence 77771. Given the nature of *P. sojae* pathotypes that exist in commercial soybean fields in South Dakota, identifying

and incorporating new sources of resistance into commercial cultivars would be necessary to manage Phytopthora root rot effectively.

To screen soybean genotypes for partial resistance to *P. sojae*, a number of methods have been adopted since the early 1980s (Schimetthenner and Bhat 1994; McBlain et al. 1991a; Tooley and Grau, 1982; Wagner at al. 1992). In direct method of inoculation, inoculum are applied on the wounded cotyledons or roots, however in some direct methods inoculation are done on non-wounded aeroponic grown plants (McBlain et al. 1991a; McBlain et al. 1991b; Tooley and Grau 1982; Wagner at al. 1992). To date, widely accepted and standardized method for screening partial resistance is the inoculum layer test and tray test (Dorrance et al. 2003; Ferro et al. 2006; Jackson et al. 2004; Lee et al. 2013; Wang et al. 2010). Besides, the rice grain inoculation method, originally developed for evaluation of tree species for *Phytophthora* resistance (Holmes and Benson, 1994) is also adopted for partial resistance evaluation (Zhang et al. 2014). Although there are several inoculation methods available for screening of soybean cultivars for partial resistance to *P. sojae* in the greenhouse, qualitative comparison of the three methods have not been done so far. Therefore our objective of the study is to (i) to compare three inoculation methods (inoculum layer test, tray test and rice grain inoculation) for screening partial resistance to *P. sojae* in the greenhouse (ii) to evaluate partial resistance to two isolates of *P. sojae* that represent two virulence pathotype (Chowdhury et al. *unpublished* [Chapter 1]) in a recombinant inbred line (RIL) population derived from the cross between cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916) (C. Ahmed and X. Gu, *unpublished*).

Materials and Methods

Source of *P. sojae* inoculum

For this study, two *P. sojae* isolates PS-15-TF3 and PS-14-F14 recovered from soil samples collected from a commercial soybean field in Turner County and Bon Homme County of South Dakota, respectively was used (R. Chowdhury et al. *unpublished* [Chapter 1]).

To recover the *P. sojae* isolates, soil baiting was conducted in the greenhouse following the procedure of Dorrance et al. (2008) by using the susceptible soybean cultivar Williams. Styrofoam cups (473 ml, Draft container corporation, Mason, MI) containing soil samples were placed in water in the greenhouse overnight at approximately 24 to 27^{o}C and then removed and placed on the greenhouse bench to allow excess water to drain for approximately 24 to 48 hours. After draining, the cups were placed in plastic bags and incubated at 22 ^{o}C in the dark for two weeks. Two weeks after incubation, each cups were planted with five seeds of cv. Williams and covered with wet coarse vermiculite. Germinated seedlings were flooded again after three days of planting and placed in greenhouse for 24 hr, then removed and placed on greenhouse bench to drain excess water. The cups were watered daily to allow for continued seedling development. The seedlings were harvested around 10 days after planting. Seedlings were collected, washed with antimicrobial soap (Equate, Bentonville, AR) in order to remove soil off the plants (Dorrance et al. 2008). In order to get rid of all the chemical and dirt, the roots were kept under running tap water for 30 min. After surface sterilization of the roots with 0.01% sodium hypchlorite solution, the roots were plated on on the PBNIC selective medium (Schmitthenner and Bhat 1994) with some modifications: 40 ml V-8 juice (Campbell's,

Camden, NJ); 0.6 g $CaCO_3$; 0.2 g Bacto yeast extract (Becton, Dickinson and Company, Erembodegem, Belgium); 1.0 g sucrose (Sigma-aldrich, St Louis, MO); 20.0 g agar (Sigma-Aldrich, St Louis, MO) in 1000 ml distilled water. After 2 to 3 days of incubation, *P. sojae* was characterized by development of a dense white mycelium with right-angle branching of coenocytic hyphae (Jackson et al. 2004). The isolates were hyphal-tipped and transferred onto fresh petri dishes containing PBNIC agar media. The cultures were observed under the microscope and mycelia that appeared to be *P. sojae* were transferred to lima bean agar (100 ml lima bean broth and 20 g agar in 1000 ml distilled water; LBA). After 3 to 5 days of incubation at 22^{o}C in dark, the oospores were readily formed on LBA agar. To verify the *P. sojae* isolates mycelial plugs were removed from the leading edges of colonies and transferred to potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ) plates since *P. sojae* does not grow on full strength PDA (Kaufmann et al. 1958).

Further confirmation was done by amplifying approximately 850 bp of the ITS (ITS4 and ITS6) region (White et al. 1990) of randomly selected 20 *P. sojae* isolates (28%) and used to query the GenBank database. Mycelia from each of the randomly selected *P. sojae* isolates were grown separately on diluted V8-juice broth and genomic DNA was extracted by using a Wizard Genomic DNA Purification Kit (Promega Inc., Madison, WI). Amplicons were send for sequencing to a DNA sequencing Service Company (Functional Biosciences Inc. Madison, WI).

To determine the pathotype of the two *P. sojae* isolates, the hypocotyl inoculation technique (Dorrance et al. 2008) was adopted on 13 differential cultivars each carrying a single *Rps* gene. The 13 differentials cultivars include included Harlon (*Rps*1a), Harosoy

13XX (*Rps*1b), Williams79 (*Rps*1c), PI 103091(*Rps* 1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2*)*, L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) (Dorrance et al. 2004) which were provided by USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC. Soybean cv. Williams was used a susceptible check. For each of the soybean differential cultivar, fifteen seedlings (7 to 10 days old) were inoculated in the hypocotyl region by injecting approximately 0.2 to 0.4 ml of mycelial slurry of prepared from an isolate into the stem using 18-guage needle. Inoculated seedlings were initially maintained for 24 hr in a moist chamber in darkness at 20 to 22^{o}C with mist applied for 60 s every 30 min. Seedlings were evaluated followed by a 7 days period with a day-night cycle of 12 hr of light and 12 hr of darkness at 25 and 23^{o}C, respectively. The plants were watered daily after inoculation. When at least seven of the ten seedlings developed an expanding necrotic brown lesion the differential was considered as susceptible. While 70% or more of the differentials inoculated with *P. sojae* survived was considered resistant (Dorrance et al. 2008). The Octal Code was determined with HaGiS spread sheet as described by Herrmann et al. (1999).

Comparison of inoculation methods

To determine an effective greenhouse-based inoculation technique to screen soybean genotypes for partial resistance to *P. sojae*, three inoculation methods; Tray test **(**Burnham et al. 2003), Inoculum layer method **(**Dorrance and Schmitthenner 2000) and Rice grain inoculation method (Holmes and Benson, 1994) were evaluated. A factorial experiment arranged in a completely randomized design with combinations of two cultivars, three inoculation methods and two *P. sojae* isolates was adopted and the

experiment was repeated twice. A single plant was considered as the experimental unit in each cup and each treatment was replicated 5 times (5 plants) in each treatment combination of cultivar, inoculation method and *P. sojae* isolate. The two soybean cultivars included cv. Surge (has *Rps*1 gene conferring tolerance to *P. sojae*) and susceptible cv. Williams with no *Rps* genes. The two isolates were selected - *P. sojae* isolate PS-15-TF3 (virulent on differentials carrying *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, *Rps*2, *Rps*3a, *Rps*3b, *Rps*3c, *Rps*4, *Rps*5, *Rps*6 and *Rps*7) and PS-14-F14 (virulent on differential carrying *Rps*7). The *P. sojae isolates* were cultured on LBA media. Plates were incubated at room temperature (25^{o}C) under dark conditions before performing greenhouse inoculations. Mycelial plugs (5 mm in diameter) were taken from the margin of the growing colony and used as inoculum for all inoculation methods tested.

For all methods, five seeds of each cultivar were planted in A4 coarse vermiculite in Styrofoam cups (473 ml, Draft container corporation, Mason, MI). The cups were placed on the greenhouse benches at 22 to 25°C under a 12-h light/dark cycle and watered on alternate days.

For tray test method (Burnham et al. 2003), soybean seedlings were grown in vermiculite-filled polystyrene containers (473 ml, Draft container corporation, Mason, MI) with bottom drainage in the greenhouse at 25°C. After 7 days, the seedlings were removed from the vermiculite and their roots were washed under running tap water. Five visually similar plants from soybean cv. Surge and cv. Williams were selected and placed on a slant board. The plants were placed on a slant board (germination paper on top of a wicking pad on a food service tray which had the raised side of one end removed). At 20 mm below the initiation of the rooting zone, a scrape wound (approximately ~ 5 mm)

were made on each seedling. Cultures of two weeks old *P. sojae* grown on soft LBA were macerated through a 50 ml syringe and approximately 0.5 ml of the mycelium-agar slurry were placed on each wound. Two trays inoculated with two *P. sojae* isolates (PS-15-TF3 and PS-14-F14) were stacked together and bound with a large rubber band and were placed in a quadrate bucket. Water (2000 ml per bucket) were added to the bottom of the bucket and changed every 2 days. The buckets were removed from the growth chamber 7 days after inoculation. Measurements (mm) were taken on the length of the lesion from the inoculation point to the top of the plant (Mideros et al. 2007).

For modified inoculum layer method **(**Dorrance and Schmitthenner 2000), two styrofoam cups (473 ml, Draft container corporation, Mason, MI) containing five plants each were prepared for the two isolates. The inoculum consisted of 2 week old *P. sojae* culture grown on lima bean agar plate at 25^{o}C. The bottom of the containers were filled with 11 cm of coarse vermiculite and wetted thoroughly. The *P. sojae* culture from the two week old lima bean agar plates were removed intact and placed on the surface of the vermiculite and covered with 2.0 cm of coarse vermiculite and were watered. Five seeds were placed on the surface of the second layer of vermiculite and covered with 2 cm of coarse vermiculite and watered again. The soybean roots were inoculated with *P. sojae* as they grew through the inoculum layer. Cups were watered thoroughly once daily. Seven days after planting, the plants were removed from the pot, roots were washed free of vermiculite and agar, and measured for lesion length from the site of root initiation toward the extended lesion on each seedling (Modified from Mideros et al. 2007).

For rice grain inoculation method (Holmes and Benson 1994), in a 250 ml Erlenmeyer flasks *P. sojae* -rice infested inoculum was prepared by autoclaving 50 grams

of long –grain rice in 36 ml of distilled water twice within a 24 h period. In between each autoclaving, the long-grain rice grains were loosened under aseptic conditions after cooling. The Erlenmeyer flasks each were inoculated with 10 pieces (0.5 cm^2) of 7 to 10 day old mycelium of *P. sojae* isolates, PS-15-TF3 and PS-14-F14, grown on LBA. The flasks that were inoculated were kept at room temperature (25^oC) for 10 to 14 days, were shaken daily to prevent the rice grains from clumping. During inoculations, about 25 g of the inoculated rice grains were placed on top of the 6.0 cm of coarse vermiculite for each sytrofoam cup. The inoculum were covered with 2.0 cm of coarse vermiculite and were watered. Five seeds were placed on the surface of the second layer of vermiculite and covered with 2 cm of coarse vermiculite and watered again. Cups were watered thoroughly once daily. Seven days after planting, the plants were removed from the pot, roots were washed free of vermiculite and agar, and measured for lesion length from the site of root initiation toward the extended lesion on each seedling (Modified from Mideros et al. 2007).

The quality assessment of the three inoculation methods was made based on the recovery of *P. sojae* from the inoculated plants. Plants were harvested 7 days after inoculation and pieces (approximately 1 cm length) were excised aseptically around the soil line and placed on the PBNIC selective medium (Schmitthenner and Bhat 1994). The entire disc of agar medium was inverted in the petri plate, covering soybean root pieces in order to limit the bacterial growth. Following a five day incubation at 25°C in dark the PBNIC plates containing mycelial plugs of *P. sojae* were examined under the microscope (40X) to characterized them. Mycelial plugs were removed from the leading edges of colonies and transferred to petri plates containing LBA. The morphological characteristics observed on PBNIC and LBA plates as described by Jackson et al. (2004) was used to

confirm for *P. sojae*. In addition, mycelial plugs of the *P. sojae* isolates mycelial plugs were removed PDA plates for confirmation (Kaufmann et al. 1958). Each of the soybean seedlings were plated separately and recovery of *P. sojae* isolates were counted as percentage.

Data was analyzed separately for the two *P. sojae* isolates. The data from the two experimental repeats were combined together for analysis after the ANOVA assumptions of normality and homogeneity of variances were satisfied. To compare the inoculation methods, linear mixed effects models was used to estimate the overall and interaction effect of cultivar, inoculation methods and *P. sojae* isolates on lesion length and mean recovery of *P. sojae* (%) using the *lme4* package (Bates et al. 2012) in R (v2.11.1; https://www.rstudio.com/; R core team 2012). As fixed effect the variables "Cultivar", "Inoculation method" and "*P. sojae* isolate" and as random effect, "experimental repeat" and "replication" were included into the model. For quality assessment of the inoculation methods, the lesion length caused by *P. sojae* and mean recovery of *P. sojae* was subjected to analysis of variance (ANOVA) for a completely randomized design for each *P. sojae* isolate and treatment means were separated using Fisher's LSD test ($P \leq 0.05$) using the Agricolae package in R (deMendiburu 2014). The relationship between lesion length caused by *P. sojae* on soybean plants 7 days after inoculation of the pathogen for different inoculation methods and recovery of *P. sojae* was quantified with Spearman rank correlation coefficients (Spearman, 1904) using R programe (v2.11.1; https://www.rstudio.com/).

Screening of the RILs for partial resistance to the two *P. sojae* isolates

A population of 100 RILs derived from a cross of cultivated *Glycine max* (cv. Surge) and wild *Glycine soja* (PI 468916) (C. Ahmed and X. Gu, *unpublished*) were used for this study. The cultivated parent 'Surge' [*Glycine max* (L.) Merr.] (Reg. no. CV-374, PI 599300) was developed by the South Dakota and Minnesota Agricultural Experiment Stations (Scott et al. 1998) and wild parent *G. soja* (PI 468916) is an annual soybean species, capable of interbreeding with domesticated soybean (Hymowitz and Singh 1987). The F1 plants from this cross were self-fertilized to produce F2 seeds. The F2 plants were self-pollinated and each line was advanced up to the F9 generation by single seed descent method. A total of 207 F8 plants were threshed individually to yield F8:9 seeds. This RIL population was developed in Dr. Xingyou Gu's Lab, Department of Agronomy, Horticulture, and Plant Science, South Dakota State University (C. Ahmed and X. Gu, *unpublished*).

In order to screen the RILs for partial resistance to *P. sojae*, the modified inoculum layer method (Dorrance et al. 2008) was adopted based on the results of the previous experiment and the the RILs were evaluated with the two *P. sojae* isolates, PS-15-TF3 and PS-14-F14). Seeds of each of the 100 RILs were planted in Styrofoam cups (473 ml, Draft container corporation, Mason, MI) filled with A4 coarse vermiculite. The styrofoam cups were arranged in a complete randomized complete design in the green house with fifteen replications (three cups with five seeds in each cup for each RILs) and each plants were considered as experimental unit. The two parents, cv. Conrad (high partial resistance) and cv. Williams (susceptible) were included as controls in each experimental repeat.

For each of the recombinant inbred line (RIL), a total of 15 plants were prepared in 3 styrofoam cups (5 plants per cup). The inoculum consisted of 2-week-old *P. sojae* cultures grown on lima bean agar in glass petri plates. The *P. sojae* -colonized agar was removed intact from the petri plate and placed 5 cm below the seed (5 soybean seeds per styrofoam cup) in course vermiculite with bottom drainage. The cups were watered to run-through twice daily. Three weeks after planting, the plants were removed from the cup. Lesion on the roots of each plants were measured separately from the site of root initiation toward the extended lesion on each seedling (Modified from Mideros et al. 2007). The experiment was performed a total of two times.

To analyze the effect of RILs, the *lme4* package (Bates et al. 2012) in R (v2.11.1; https://www.rstudio.com/; R core team, 2012) was used to perform linear mixed effects models. Into the model, "Genotype (RILs)" was entered as a fixed effect and "Experimental repeat" as random effect. ANOVA assumptions of normality and homogeneity of variances were satisfied and data from two runs of each experiment were combined together for analysis. Data were subjected to analysis of variance (ANOVA) for a completely randomized design and means for each genotype were separated using Fisher's protected least significant differences (LSD) ($P \leq 0.05$) in R using the Agricolae package in R (deMendiburu 2014). Data was analyzed separately for te two *P. sojae* isolates.

Results

Source of *P. sojae* inoculum

White dense mycelia appeared on the PBNIC agar media for the two isolates of *P. sojae* following 2 to 3 days after transferring on the agar plates and the isolates cover the whole agar plates within 7 to 10 days. The *P. sojae* mycelium on the PBNIC media was

characterized by coenocytic hyphae, high branching with curved tips and right angle branching (Jackson et al. 2004). On LBA agar plates oospores of *P. sojae* isolates were formed within 3 to 4 days.

Molecular confirmation of the *P. sojae* isolates was performed by analyzing the ITS sequence and the ITS sequences from the two *P. sojae* isolates matched the ITS sequence of the *Phytophthora sojae* isolate SDSO_9-72 (Accession # KU211500.1) with identities = 834/834 (100%) and gaps = 0/ 834 (0%). The ITS sequences of the *P. sojae isolates* (PS-15-TF3 and PS-14-F14) generated in this study are deposited in the GenBank under accession numbers KX668417 and KX668418.

The *P. sojae* isolate PS-15-TF3 was assigned by the virulence formula 77771 given that the isolate showed susceptible reaction on all the 13 soybean differentials (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, *Rps*2, *Rps*3a, *Rps*3b, *Rps*3c, *Rps*4, *Rps*5, *Rps*6 and *Rps*7) and The *P. sojae* isolate PS-14-F14 was assigned by virulence formula 00001(formally Race 1) given that the isolate showed susceptible reaction on only one differential (*Rps*7).

Comparison of inoculation methods

Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as cultivar, inoculation method ($P > 0.05$) on lesion length and recovery of *P. sojae* isolates (%). For *P. sojae* isolate PS-15-TF3, a significant two way cultivar x inoculation method interaction was observed for lesion length ($\chi^2 = 8.11$, df =2, $P < 0.001$) indicating that the cultivar and inoculation methods significantly influenced the lesion length caused by *P. sojae* 7 days after inoculation. For *P. sojae* isolate PS-14-F14, the interaction effect of cultivar and inoculation method was not significant for lesion length ($\chi^2 = 3.30$, df =2, $P = 0.19$).

However, the type of cultivar had a significant effect on the lesion length (χ^2 =28.57, df =1, *P* <0.001) (Table 3.1).

Irrespective of the *P. sojae* isolates used for inoculation, there was no two way cultivar x inoculation method interaction for recovery of *P. sojae* isolates (χ^2 =3.73, df =2, *P* = 0.15 for PS-15-TF3 and χ^2 =0.89, df =2, *P* = 0.63 for PS-14-F14). However, inoculation methods had a significant effect on the recovery of *P. sojae* (χ^2 =164.71, df =1, *P*<0.001 for PS-15-TF3 and χ^2 =94.56, df =1, *P* <0.001 for PS-14-F14) (Table 3.1).

Effect of lesion length on cultivar and inoculation methods

***P. sojae* isolate PS-15-TF3**: The lesion length caused by PS-15-TF3 on soybean plants varied when different inoculation methods were used. The soybean plants inoculated with rice grain inoculation method had significant higher lesion length as compared to inoculum layer test and tray test (LSD= 4.20, *P*<0.001). On cv. Surge, the lesion length was higher by 16% and 20% for rice grain inoculation method as compared to the inoculum layer test and tray test methods respectively. On cv. Williams, the lesion length was higher by 2% and 27% for rice grain inoculation method as compared to inoculum layer test and tray test respectively. However, the overall lesion length was higher on plants of cv. Williams as compared to cv. Surge irrespective of the inoculation methods used (Table 3.2).

***P. sojae* isolate PS-14-F14**: The soybean plants inoculated with rice grain inoculation method had significant higher lesion length as compared to inoculum layer test and tray test (LSD= 5.40, *P*<0.001). For instance, for cv. Surge, the lesion length was higher by 18% for rice grain inoculation method as compared to inoculum layer test and tray test. On cv. Williams lesion length was higher by 13% and 5% for rice grain

inoculation method as compared to inoculum layer test and tray test, respectively. However, the overall lesion length was more on plants of cv. Williams as compared to cv. Surge irrespective of the inoculation methods used (Table 3.2).

Effect of recovery of *P. sojae* (%) on cultivar and inoculation methods

***P. sojae* isolate PS-15-TF3**: The mean recovery of *P. sojae* (%) differed significantly (LSD=6.36, $P < 0.0001$) among the inoculation methods (Table 3.2). On cv. Surge, the lowest re-isolation percentage was obtained from plants inoculated with rice grain inoculation method (26.5%) followed by tray test (72.5%), while the highest percentage of re-isolation was obtained from plants inoculated with the inoculum layer method (94.5%) (Table 3.2). Similar trend was observed for cv. Williams, the lowest re-isolation percentage was obtained from plants inoculated with rice grain inoculation method (27%) followed by tray test (70%), while the highest percentage of re-isolation was obtained from plants inoculated with inoculum layer method (89.5%) (Table 3.2). No pathogen was isolated from the control plant inoculated with non infested agar plug (in the inoculum layer or tray test method) or rice grain (data not presented).

***P. sojae* isolate PS-14-F14**: The mean recovery of *P. sojae* (%) differed significantly (LSD=6.47, $P < 0.0001$) among the inoculation methods (Table 3.2). On cv. Surge, recovery percentage was lowest in plants inoculated with rice grain inoculation method (58.1%) followed by tray test (79.5%), while the highest percentage of re-isolation was obtained from plants inoculated with the inoculum layer method (89.0%) (Table 3.2). On cv. Williams, rice grain inoculation method showed the lowest re-isolation percentage (56.0%) followed by tray test (77.5%), while the highest percentage of re-isolation was obtained from plants inoculated with inoculum layer methods (90.5%) (Table 3.2). No

pathogen was isolated from the control plant inoculated with non-infested agar plug or rice grain (data not presented).

Upon performing Spearman rank correlations between mean lesion length at 7 days after inoculation and recovery of *P. sojae* (%), we observed a moderate negative and highly significant correlation coefficient (ρ = -0.57) for rice grain inoculation method (P = 0.0001). For tray test, the rank correlation coefficient was low (ρ = -0.30) and significant at P = 0.02. For inoculum layer test, the rank correlation coefficient was low (ρ = -0.11) and significant at P = 0.05. Based on the recovery of *P. sojae* isolates (%) and its correlation with lesion length at 7 days after inoculation, inoculum layer test method was implemented for evaluation of partial resistance in the RIL population.

Evaluation of RILs for partial resistance

Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as, *P. sojae* isolates and genotypes (P> 0.05) on the overall lesion development by *P. sojae* isolates (data not presented). Significant differences in lesion length were observed for the lines inoculated with the isolate PS-15-TF3 (χ^2 =1391.30, df =103, P <0.001) and PS-14-F14 (χ^2 =1456.00, df =103, P <0.001) as compared to the parents and checks (Conrad and Williams).

***P. sojae* isolate PS-15-TF3**: Partial resistance level for each RIL was assessed based on lesion length 21 days after *P. sojae* inoculation. The mean lesion lengths of 100 RILs were continuously distributed between 1.0 to 72.1 mm and there was significant difference in lesion length among the RILs (P < 0.0001). The mean lesion lengths were 77.60, 65.10, 25.05, and 43.10 mm for cv. Surge, Williams, Conrad, and *Glycine soja*, respectively. Sixty three out of 100 of the RILs had significantly shorter lesion length (LSD

= 4.06, *P* <0.001) as compared to *Glycine soja* when inoculated with *P. sojae* isolate PS-15-TF3 (Table 3.3).

***P. sojae* isolate PS-14-F14**: The mean lesion lengths of 100 RILs ranged between 0.5 to 68.0 mm and there was significant difference in lesion length among the RILs (*P* < 0.0001). Mean lesion lengths for cv. Surge, Williams, Conrad, and *Glycine soja* were 33.53, 65.50, 21.75, and 25.60 mm respectively. The mean lesion lengths were significantly smaller in *Glycine soja* than cv. Surge (LSD= 1.53, *P* < 0.0001) over the experiments, and the mean lesion length of all the RILs were 23.24 mm was intermediate between the two parents (Table 3.3). For *P. sojae* isolate PS-14-F14, 39 of the RILs had significantly shorter lesion length (LSD = 3.00 and *P* = <0.001) as compared to *Glycine soja* (Table 3.4).

Discussion

In our study, three inoculation methods were assessed to screen soybean genotypes for partial resistance to *P. sojae* in the greenhouse. Based on the recovery of *P. sojae* and its correlation with lesion length caused by the *P. sojae* isolates at 7 days after inoculation, inoculum layer method was adopted for evaluation of partial resistance in the RIL screening experiment. By using the inoculum layer method, 100 RILs derived from the cross between cv. Surge and *Glycine soja* were evaluated for partial resistance to two isolates of *P. sojae* (PS-15-TF3 and PS-14-F14). Sixty three of the RILs had significantly shorter lesion length as compared to the moderately resistant parent *Glycine soja* when inoculated with *P. sojae* isolate PS-15-TF3. For PS-14-F14, 39 of the RILs had significantly shorter lesion length as compared to the moderately resistant parent *Glycine soja.* When inoculated with either *P. sojae* isolate PS-15-TF3 or *P. sojae* isolate PS-14-

F14, 9 of the RILs had relatively shorter lesion length (lesion size 0-5 mm) than the moderately resistant parent *Glycine soja*.

While comparing the three inoculation methods (tray test, inoculum layer method and rice grain inoculation method) significant differences were observed based on lesion development and recovery of *P. sojae* isolates (%) after 7 days of inoculation. Based on our study, we adopted inoculum layer method for partial resistance evaluation because we found higher recovery of *P. sojae* despite observing lower lesion length on inoculation soybean plants as compared to tray test and rice grain inoculation method. In general, inoculum layer method is tedious and costly since it requires handling of a large number of agar plates (Stewart and Robertson 2010). Additionally, the inoculum layer method may have limitation in using of multiple isolates in a single test (Stewart and Robertson 2010). However, despite the disadvantages, the inoculum layer method has been suitably used for screening *P. sojae* for partial resistance in several studies and resistant genotypes identified. For example, in the study by Jia and Kurle (2008), 69 PIs were used for evaluation of partial resistance to *P. sojae* races 7 (conferring resistance to *Rps*1a, *Rps*2, *Rps*3a, *Rps*3c, *Rps*4, *Rps*5, *Rps*6, *Rps*7and race 25 (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, *Rps*7) using the inoculum layer method. They found twelve PIs had the same level of partial resistance as Conrad to both *P. sojae* races 7 and 25, suggesting for the evaluation of the parents of the line that had lowest lesion size for both the *P. sojae* races.

Between the soybean parents used in this study to screen soybean RILs for partial resistance to *P. sojae*, *G. soja* is capable of interbreeding with domesticated soybean (Hymowitz and Singh 1987) and several researchers have discovered the existence of genetic diversity present in *G. soja* which is absent in the domesticated soybean species

(Keim et al. 1989; Maughan et al. 1995). This knowledge of diversity has been subjected to genetic mapping studies in soybean. For example, a genetic map was developed from the cross between the *G. soja* line PI 468916 and the soybean experimental line A81-356022 with more than 1000 molecular markers (RFLP and SSR) (Shoemaker and Olson 1993). Later on, Wang et al. (2001) discovered that the *G. soja* parent (PI 468916) used for map construction has two QTLs that confer resistance to SCN race 3. However, information on the potentiality for having new sources of resistance for *P. sojae* in *G. soja* (PI 468916) is still lacking. In this study, 100 RILs derived from the cross between cv. Surge and *Glycine soja* were evaluated for partial resistance to *P. sojae* in the greenhouse and resistant RILs identified. Nevertheless, we identified 9 RILs that had comparatively smaller lesion length (lesion size 0-5mm) than *Glycine soja* when inoculated with either PS-15-TF3 or PS-14-F14. These 9 RILs may be used as potential sources of partial resistance to *P. sojae* for developing commercial soybean varieties in future, partially because they exhibited potential resistance to the pathogen when inoculated with an isolate that was virulent on all 13 soybean differentials. In addition, these RILs might be evaluated further for additional Quantiative Trait Loci (QTL) sources for partial resistance associated with lesion length. Using lesion length, previous studies have identified a number of QTLs during evaluation of soybean germplasm for partial resistance to *P. sojae* (Burnham et al. 2003; Lee et al. 2013; Tucker et al. 2010; Wang et al. 2012;). For example, Tucker et al. (2010) evaluated a interspecific RIL population of 296 individuals that were derived from the cross of *G. max* V71-370 and *G. soja* PI 407162. They identified three QTLs on chromosomes 16, 20, and 18 accounted for 32, 42, and 22%, respectively, of the phenotypic variation. Similarly, it would be important to identify QTLs associated with the 9 RILs

conferring resistance to *P. sojae* in our study and this information is important for breeders to be able to map and develop soybean varieties with field resistance to *P. sojae*.

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Table 3.1 *P* values for main and interaction effect of cultivars (Surge and Williams) and inoculation methods on lesion length caused by *P. sojae* isolates PS-15-TF3 and PS-14-F14 on soybean plants and recovery percentage of *P. sojae*.

***P. sojae* isolates**	**Variables**	**Effects[a,d]**		
		Cultivar	Methods	Cultivar x Methods
PS-15-TF3	Lesion length	0.001	<0.001	0.017
	Recovery of *P. sojae* (%)	ns	<0.001	ns
PS-14-F14	Lesion length	<0.001	ns	ns
	Recovery of *P. sojae (*%)	ns	<0.001	ns

[a] *P* values associated with the two parameters (Cultivar, methods) was determined using the likelihood ratio test [in the *lme4* (Bates et al. 2012) package] in which a "full" model containing fixed effects was compared against a "reduced" model without the fixed effects. For the likelihood ratio test, the fixed effect was considered significant if the difference between the likelihood of the full and reduced models was significant at $P \leq 0.05$.

[b] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R.

[c] ns=not significant at $P \geq 0.05$.

Table 3.2 Mean lesion length caused by *P. sojae* on plants of two soybean cultivars of evaluated for partial resistance to *P. sojae* using three inoculation methods in the greenhouse and recovery of *P. sojae*

***P. sojae* isolates[a]**	**Cultivars[b]**	**Inoculation methods**	**Lesion length (mm)[c,d]**	**Recovery percentage[d,e]**
PS-15-TF3	Surge	Inoculum layer test	39.4 cd	94.5 a
		Tray test	37.5 d	72.5 b
		Rice grain inoculation	47.0 b	26.5 c
	Williams	Inoculum layer test	42.5 bcd	89.5 a
		Tray test	43.8 bc	70.0 b
		Rice grain inoculation	60.5 a	27.0 c
PS-14-F14	Surge	Inoculum layer test	31.4 c	89.0 a
		Tray test	31.3 c	79.5 b
		Rice grain inoculation	38.3 b	58.1 c
	Williams	Inoculum layer test	38.6 b	90.5 a
		Tray test	42.2 ab	77.5 b
		Rice grain inoculation	44.5 a	56.0 c

[a] Data for the two *P. sojae* isolates (PS-15-TF3 and PS-14-F14) were analyzed separately.

[b] Surge has tolerance to *P. sojae* (*Rps* 1a), Williams is moderately susceptible to *P. sojae*

[c] Lesion lengths were measured from the site of root initiation toward the extended lesion on each seedling (Mideros et al. 2007)

[d] Data from the two experiments were combined together after satisfying the homogeneity of variances assumption and values represents the means of two experiments with ten replication each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05)

[e] Recovery of *P. sojae isolates* were counted as percentage.

Table 3.3 Descriptive statistics of mean lesion length of parents, checks, and 100 recombinant inbred lines (RILs) of the *Glycine max* X *G. soja* (PI 468916) population.

Pathotypes	Trait	Parents and Check[a,b]				RIL population			
		Surge	*Glycine soja*	Conrad	Williams	*N*[c]	Mean	Range	SD
PS-15-TF3	Lesion length (mm)	77.6 a	43.1 c	25.0 d	65.1 b	100	32.9	1.0-72.1	16.9
PS-14-F14	Lesion length (mm)	33.5 b	25.6 c	21.7 d	65.5 a	100	23.2	0.5-68.0	14.8

[a] Conrad has high partial resistance and Williams in moderately susceptible

[b] Means are separated within rows and numbers followed by same letter are not significantly different based on Fisher's least significant difference test (R program v2.11.1; https://www.rstudio.com/)

[c] Number of recombinant inbred lines evaluated

Table 3.4 Mean lesion length of recombinant inbred lines (RILs), checks (Conrad and Williams) and parents [*Glycine max* (cv. Surge), *Glycine soja*] inoculated with *P. sojae* isolate PS-15-TF3 and *P. sojae* isolate PS-14-F14.

PS-15-TF3		PS-14-F14	
RILs[a]	Mean lesion length(mm)[b]	RILs[a]	Mean lesion length(mm)[b]
cv. Surge	77.6 a	RIL 201	67.9 a
RIL 206	71.7 b	Williams	65.5 a
RIL 189	68.8 bc	RIL 20	60.3 b
Williams	65.1 cd	RIL13	58.2 b
RIL 80	63.1 de	RIL49	50.8 c
RIL 17	60.9 ef	RIL17	50.7 c
RIL 78	60.8 ef	RIL88	49.9 cd
RIL 40	60.6 efg	RIL75	47.7 de
RIL 90	59.0 fg	RIL11	46.6 ef
RIL 81	58.6 fg	RIL71	46.2 ef
RIL 97	58.2 fg	RIL6	45.7 ef
RIL 49	57.8 fg	RIL81	44.6 fg
RIL 82	56.9 fgh	RIL110	42.6 gh
RIL 112	56.6 gh	RIL9	40.8 hi
RIL 74	53.4 hi	RIL69	39.2 ij
RIL 126	53.1 hij	RIL106	37.2 j
RIL 122	52.2 ijk	RIL16	33.9 k
RIL 85	51.3 ijkl	RIL67	33.7 k
RIL 88	51.1 ijkl	cv. Surge	33.5 kl
RIL 19	50.6 ijklm	RIL189	32.4 klm
RIL 114	49.3 ijklmn	RIL119	32.1 klmn
RIL 71	48.6 klmno	RIL19	31.3 klmno
RIL 6	48.5 klmno	RIL156	31.2 klmno
RIL 120	48.1 klmno	RIL61	30.6 lmnop

[a] Recombinant inbred lines

[b] Lesion lengths were measured from the site of root initiation toward the extended lesion on each seedling (Mideros et al. 2007). Values are the means of two experiments with ten replication each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05) (R program v2.11.1; https://www.rstudio.com/)

Table 3.4 (contd.) Mean lesion length of recombinant inbred lines (RILs), checks (Conrad and Williams) and parents (*Glycine max*, *Glycine soja*) inoculated with *P. sojae* isolate PS-15-TF3 and *P. sojae* isolate PS-14-F14.

PS-15-TF3		PS-14-F14	
RILs[a]	Mean lesion length(mm)[b]	RILs[a]	Mean lesion length(mm)[b]
RIL 75	48.1 klmno	RIL105	30.4 mnopq
RIL 156	47.3 lmnop	RIL48	29.9 mnopqr
RIL 115	46.6 mnopq	RIL89	29.7 mnopqrs
RIL 83	46.6 mnopq	RIL87	29.3 nopqrst
RIL 128	45.7 nopqr	RIL44	28.3 opqrstu
RIL 110	44.6 opqrs	RIL78	28.3 opqrstu
RIL 14	43.6 pqrst	RIL70	28.3 opqrstu
RIL 70	43.1 qrstu	RIL10	28.1 pqrstuv
Glycine soja	43.1 qrstu	RIL109	28.1 pqrstuv
RIL 124	42.5 rstu	RIL123	27.5 qrstuvw
RIL 69	42.2 rstu	RIL127	27.3 rstuvw
RIL 9	41.4 stu	RIL3	27.3 rstuvw
RIL 87	40.6 stuv	RIL14	27.1 rstuvwx
RIL 4	39.9 tuvw	RIL8	26.9 rstuvwx
RIL 106	39.6 tuvw	RIL83	26.8 stuvwxy
RIL 92	39.4 uvw	RIL120	26.6 tuvwxyz
RIL 48	36.5 vwx	RIL98	26.4 tuvwxyz
RIL 61	36.4 wx	RIL93	26.2 uvwxyzA
RIL 174	33.9 xy	RIL95	26.1 uvwxyzA
RIL 11	33.2 xyz	RIL116	26.1 uvwxyzA
RIL 16	32.7 xyzA	RIL97	25.8 uvwxyzAB
RIL 119	32.6 xyzA	RIL90	25.8 uvwxyzAB
RIL 107	32.5 xyzA	*Glycine soja*	25.6 uvwxyzAB
RIL 99	31.9 yzAB	RIL72	25.5 uvwxyzAB
RIL 109	31.4 yzABC	RIL76	25.3 uvwxyzABC
RIL 127	31.3 yzABC	RIL125	25.3 uvwxyzABC
RIL 98	31.3 yzABC	RIL15	25.2 vwxyzABCD

[a] Recombinant inbred lines

[b] Lesion lengths were measured from the site of root initiation toward the extended lesion on each seedling (Mideros et al. 2007). Values are the means of two experiments with ten replication each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05) (R program v2.11.1; https://www.rstudio.com/)

Table 3.4 (contd.) Mean lesion length of recombinant inbred lines (RILs), checks (Conrad and Williams) and parents (*Glycine max*, *Glycine soja*) inoculated with *P. sojae* isolate PS-15-TF3 and *P. sojae* isolate PS-14-F14.

PS-15-TF3		PS-14-F14	
RILs[a]	Mean lesion length(mm)[b]	RILs[a]	Mean lesion length(mm)[b]
RIL 84	30.4 yzABCD	RIL92	25.1 vwxyzABCD
RIL 89	30.3yzABCDE	RIL99	24.9 wxyzABCD
RIL 91	29.9 yzABCDEF	RIL4	24.8 wxyzABCD
RIL 116	29.5 zABCDEF	RIL66	24.7 wxyzABCDE
RIL 44	29.4 zABCDEF	RIL128	24.5 wxyzABCDEF
RIL 123	29.0 ABCDEFG	RIL73	24.3 xyzABCDEF
RIL 73	28.9 ABCDEFGH	RIL45	24.2 xyzABCDEF
RIL 96	28.9 ABCDEDGHI	RIL51	24.2 xyzABCDEF
RIL 125	28.4 BCDEFGHI	RIL21	23.8 yzABCDEFG
RIL 3	28.4 BCDEFGHI	RIL12	23.7 zABCDEFG
RIL 15	28.4 BCDEFGHIJ	RIL85	23.7 zABCDEFG
RIL 68	27.9 BCDEFGHIJ	RIL101	23.6 ABCDEFG
RIL 102	27.9 BCDEFGHIJK	RIL114	22.9 BCDEFG
RIL 66	27.6 CDEFGHIJKL	RIL96	22.3 CDEFG
RIL 95	27.5 CDEFGHIJKL	RIL2	22.2 DEFGH
RIL 105	27.0 DEFGHIJKLM	Conrad	21.7 EFGHI
RIL 12	26.8 DEFGHIJKLM	RIL86	21.7 FGHI
RIL 104	26.5 DEFGHIJKLMN	RIL102	21.6 FGHI
RIL 45	26.3 EFGHIJKLMN	RIL40	21.6 FGHI
RIL 51	26.3 EFGHIJKLMN	RIL206	20.8 GHI
RIL 72	26.2 FGHIJKLMN	RIL100	19.2 HIJ
RIL 2	26 FGHIJKLMNO	RIL65	19.0 IJ
Conrad	25.0 GHIJKLMNOP	RIL91	18.8 IJ
RIL 20	24.9 HIJKLMNOP	RIL74	17.3 JK
RIL 67	24.8 IJKLMNOP	RIL196	16.8 JK
RIL 93	24.3 JKLMNOP	RIL84	15.8 K
RIL 77	23.8 KLMNOPQ	RIL7	11.9 L
RIL 76	23.7 LMNOPQ	RIL107	11.0 L

[a] Recombinant inbred lines

[b] Lesion lengths were measured from the site of root initiation toward the extended lesion on each seedling (Mideros et al. 2007). Values are the means of two experiments with ten replication each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05) (R program v2.11.1; https://www.rstudio.com/)

Table 3.4 Contd…Mean lesion length of recombinant inbred lines (RILs), checks (Conrad and Williams) and parents (*Glycine max*, *Glycine soja*) inoculated with *P. sojae* isolate PS-15-TF3 and *P. sojae* isolate PS-14-F14.

PS-15-TF3		PS-14-F14	
RILs[a]	Mean lesion length(mm)[b]	RILs[a]	Mean lesion length(mm)[b]
RIL 100	23.4 MNOPQ	RIL103	8.0 M
RIL 101	23.4 MNOPQ	RIL77	6.5 MN
RIL 201	22.4 NOPQR	RIL64	6.4 MN
RIL 65	21.9 OPQRS	RIL68	6.3 MNO
RIL 10	21.8 PQRS	RIL174	5.6 MNOP
RIL 42	21.6 PQRS	RIL115	4.6 NOPQ
RIL 21	20.2 QRS	RIL55	4.6 NOPQ
RIL 13	19.3 RS	RIL80	4.4 NOPQ
RIL 86	19.1 RST	RIL126	3.7 NOPQR
RIL 103	18.6 RSTU	RIL124	3.3 OPQRS
RIL 55	18.1 STU	RIL104	3.2 PQRS
RIL 7	15.2 TU	RIL18	3.1 PQRS
RIL 196	14.8 U	RIL118	2.8 PQRS
RIL 5	6.35 V	RIL112	2.8 PQRS
RIL 121	4.7 VW	RIL108	2.8 PQRS
RIL 18	4.6 VW	RIL160	2.8 PQRS
RIL 64	4.5 VW	RIL122	2.7 PQRS
RIL 108	3.9 VW	RIL121	2.7 PQRS
RIL 160	3.1 VW	RIL5	2.4 QRS
RIL 79	2.8 VW	RIL42	2.3 QRS
RIL 113	2.6 VW	RIL113	2.2 QRS
RIL 118	2.2 W	RIL62	1.9 QRS
RIL 1	1.6 W	RIL79	1.6 QRS
RIL 8	1.2 W	RIL1	1.2 RS
RIL 62	1.1 W	RIL82	0.5 S

[b] Lesion lengths were measured from the site of root initiation toward the extended lesion on each seedling (Mideros et al. 2007). Values are the means of two experiments with ten replication each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05) (R program v2.11.1; https://www.rstudio.com/)

[a] Recombinant inbred lines

CHAPTER 4

Title: Interaction between *Phytophthora sojae* and Soybean Cyst Nematode on Soybean (*Glycine max*)

Chowdhury, R. N.[1], Acharya, K.[2], Byamukama, E.[1], Kontz, B.[1], Okello, P. N.[1], and Mathew, F. M.[1] 201X. Interaction between *Phytophthora sojae* and soybean cyst nematode on soybean (*Glycine max*).

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Abstract

Phytophthora sojae and soybean cyst nematode (SCN) are important pathogens of soybean. Although these pathogens infect soybean roots, there is limited evidence of interaction between them. The objective of this study was to examine the interaction between SCN and *P. sojae* on soybean in the greenhouse. Seeds of 4 soybean cultivars [Jack, Surge, Williams 82, Williams] were pre-germinated and placed in cone-tainers containing steam pasteurized sand-clay mixture. The experiment was set up in a completely randomized design with five replications, and performed twice. Two *P. sojae* isolates were used in this study that represented 2 different virulence pathotypes. For each isolate,

soybean plants were inoculated with one of the treatments – SCN, *P. sojae*, and combination of *P. sojae* and SCN. To inoculate with *P. sojae*, mycelial plugs were placed adjacent to the soybean plants. The plants were placed in the mist chamber for 48 h, and then appropriate treatments were inoculated with SCN. After 35 days, stem length, root length, plant weight, root weight, lesion length, and SCN population were recorded. On all soybean cultivars, the lesion length caused by *P. sojae* increased in the presence of SCN. However, SCN population was reduced by*P. sojae* for the two isolates.

Keywords: *Phytophthora*, SCN, soybean

Introduction

Phytophthora root and stem rot caused by the oomycete *Phytophthora sojae*, Kaufmann and Gerdemann, is one of the major yield-limiting diseases of soybean (*Glycine max* (L.) Merrill) in the United States. The disease caused an approximate loss of $338 million (93 thousand metric tons) to producers according to the 2014 market values for soybean (USDA - National Agricultural Statistics Service; https://www.nass.usda.gov/; Bradley et al. 2014). *Phytophthora sojae* can infect soybean plants at all growth stages throughout the growing season when environmental conditions are favorable. If infection occurs during the vegetative growth stages, soybean seedlings develop typical symptoms of pre-and post-emergence damping-off and root rot. At reproductive growth stages of soybean, taproots of the infected soybean plants become brown and the brown discoloration extends up the stem causing plant death (Schmitthenner 1985).

Phytophthora sojae overwinters as oospores in crop residue or soil which serves as the primary inoculum. Under suitable moisture and temperature conditions, the dormancy

of oospores is broken. Sporangia are produced at the tips of hyphae which release zoospores under warm temperatures (25 to 30°C) and flooded soil conditions (Schmitthenner 1985). The zoospores are attracted by exudates from roots of the soybean plants (Morris et al. 1998). They swim to the host root and encyst on the root surface. The germinating zoospores produce an appressorium at the end of germ tube, which enables the pathogen to penetrate into the root tissue. After entry into the root tissue, *P. sojae* produces a haustorium for uptake of nutrients from the host cells and colonizes the soybean plant (Schmitthenner 1985).

The variability of *P. sojae* has been described based on the compatible (susceptible) and incompatible (resistant) reactions on soybean differential lines containing a unique resistance gene (*Rps*). At this time, more than 55 races of *P. sojae* have been described (Grau et al. 2004). However, the presence of one *Rps* gene incorporated in the 14 soybean differentials (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, *Rps*3a, *Rps*6, *Rps*7 and *Rps*8) can increase the possibility of numerous races continuously emerging in the pathogen. Thus, instead of races, pathotypes and octal codes are used to define virulence phenotypes of *P. sojae* (Dorrance et al. 2005; Herrmann et al. 1999; Robertson et al. 2009). In the United States, numerous surveys have been conducted to determine the pathotype of *P. sojae* population prevalent in the soybean production regions of the country. For example, in the study by Stewart et al. (2016), *P. sojae* isolates were recovered from 17, 36 and 19 field locations in Iowa, Ohio and South Dakota respectively to study the pathotype and genetic diversity within and among populations of *P. sojae* in the three different states. Based on the Shannon diversity index (Spellerberg and Fedor 2003) that measures the relative differences in pathotypes among the isolates, the pathotype diversity was highest for Ohio

(3.37) followed by South Dakota (2.76) and Iowa (2.15). However, when genetic diversity was studied with individual fields in Iowa (5 fields), Ohio (6 fields) and Missouri (1 field), Shannon diversity was ranged from 1.61 to 2.48 for Ohio, 3.01 for Missouri and less than 1.00 for the fields in Iowa based on the analyses of total 108 *P. sojae* isolates recovered from the three states. In addition, *P. sojae* pathotypes were identified that were virulent on all 13 soybean differentials, which is not surprising given the complex nature of the pathogen (Stewart et al. 2016). Under field conditions, among the factors that potentially have a role in affecting the disease severity caused by *P. sojae* on soybean, soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe is possibly important.

Soybean cyst nematode is one of the most economically important pest of soybean in the United States and accounts for $1 billion in revenue losses annually (Chen 2011; Wrather et al. 2009). Given both the pathogens are capable of infecting soybean roots, there are possibilities of interaction between the two pathogens thus affecting the overall growth of soybean. For example, in a study by Adeniji et al. (1975), an additive interaction between SCN and *P. sojae* was observed. The root rot severity (measured by a disease rating scale of 1-4; Adeniji et al. 1975) caused by *P. sojae* race 1 (showing virulent reaction on differential with *Rps*7 gene) was higher on a susceptible soybean cultivar (‘Corsoy’) in the presence of SCN race 3 (*H. glycines* (HG) type 0) when compared to the root rot severity caused by *P. sojae* by itself on ‘Corsoy’. In a study by Kaitany et al. (2000), the incidence of *P. sojae* at high and low fumigated SCN condition was assessed and it was observed that *P. sojae* incidence can increase on soybean plants stressed from SCN infestation.

In South Dakota, the distribution of SCN overlaps with that of Phytophthora root and stem rot in the soybean producing counties based on a survey of 200 commercial soybean fields in 2014 (F. Mathew, *unpublished*). At this time, there is no information available on the yield loss due to the co-existence of SCN and *P. sojae* on soybean plants in these fields. However, it is possible that the soybean farmers are experiencing more yield losses from the two pathogens together as compared to losses from either of the pathogens by itself. In order to manage SCN, most soybean farmers in the North Central United States including in South Dakota use cultivars with resistance derived from PI 88788, Peking or PI 437654 (Joos et al. 2013; Mitchum 2016; Tylka and Mullaney 2015). In these commercial SCN resistant varieties, the genes *Rps*1a, *Rps*1c and *Rps*1k are commonly deployed in the form of partial resistance to manage Phytophthora root and stem rot in South Dakota (Dorrance et al. 2003). However, shifts in *P. sojae* pathotypes have been implied in a recent study characterizing the pathotype diversity of *P. sojae* in commercial soybean fields in South Dakota and about 4% of the isolates were able to produce virulent reaction on all 13 soybean differentials (R. Chowdhury and E. Byamukama, *unpublished*). In this study, we hypothesized that the presence of SCN can not only increase the lesion length of the disease caused by *P. sojae* complex pathotypes (e.g. PS-15-TF3), but the co-infection of the two pathogens can affect soybean growth during the infection process. To test the hypothesis, a *P. sojae* isolate (PS-15-TF3) that is virulent on all 13 soybean differentials is compared with a *P. sojae* isolate (PS-14-F14) representing Race 1 (showing virulent reaction on differential carrying *Rps*7) during their individual interaction with SCN on soybean in the greenhouse. The specific objectives of this study were (i) to determine whether the interaction between SCN and *P. sojae* can affect soybean plants in greenhouse;

(ii) to evaluate the lesion length caused by *P. sojae* on soybean in presence of SCN in the greenhouse; and (iii) to evaluate the SCN development on soybean in the presence of *P. sojae* in the greenhouse.

Materials and methods

***Phytophthora sojae* isolation, identification and pathotype characterization**

For *P. sojae* inoculum, two isolates PS-15-TF3 and PS-14-F14 were recovered from soil samples collected from a commercial soybean field in Turner County, SD and in Bon Homme County, SD respectively (R. Chowdhury et al. *unpublished* [Chapter 1]).

To recover *P. sojae isolates* from the soil samples, a soil baiting method was used (Dorrance et al. 2008). Styrofoam cups (473 ml, Draft container corporation, Mason, MI) containing soil samples were flooded for 24 h using tap water, drained, and air dried until the moisture content reached a matric potential of approximately –300 mb. The cups were placed in polyethylene bags and incubated at 22^{o}C for a total of 2 weeks. Following the incubation period at 22^{o}C, five seeds of the susceptible soybean cv. Williams (provided by Dr. Anne E. Dorrance, the Ohio State University, Columbus, OH) were placed on top of the soil in the cups and covered with wet coarse vermiculite (Therm-O-Rock, New Eagle, PA). Three days after planting of cv. Williams, the cups were flooded again for 24 h and placed on greenhouse benches to drain the water. Ten days after planting, soybean seedlings were harvested; each seedling was rinsed under tap water, and washed with antimicrobial soap (Equate, Bentonville, AR) in order to remove soil off the plants (Dorrance et al. 2008). After soil was removed, roots were kept under the running tap water for 30 min. Soybean roots were disinfested with 0.05% sodium hypochlorite for 30 s, washed in sterile distilled water and air dried on a sterile paper towel. Small pieces of the

root (approximately 1 cm) were excised aseptically around the soil line and placed on the selective modified PBNIC medium (40 ml V8 juice (Campbell Soup Company, Camden, NJ), 0.6 g $CaCO_3$ (Sigma-Aldrich, St Louis, MO), 0.2 g Bacto Yeast extract (Becton, Dickinson and Company, Erembodegem, Belgium), 1.0 g sucrose (Sigma-Aldrich, St Louis, MO), 20.0 g agar (Sigma-Aldrich, St Louis, MO) in 1000 ml distilled water) (Schmitthenner and Bhat 1994). The PBNIC petri plates were incubated for 3 to 4 days at 22±2^{o}C in dark. The whole disc of agar media were inverted to limit bacterial contamination.

To purify *P. sojae* cultures, mycelial plugs were removed from the leading edges of colonies in the PBNIC plates and transferred to petri plates containing lima bean agar (100 ml lima bean broth and 20 g agar in 1000 ml distilled water; LBA). After 2 to 3 days of incubation at 22^{o}C and in dark, all the colonies were examined with a microscope (at 40X magnification) for characteristic appearance of mycelium and for oospore formation. After 3 days, mycelial plugs were removed from the leading edges of colonies and transferred to potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ) plates for the confirmation of *P. sojae*, since the pathogen does not grow on full strength PDA (Kaufmann et al. 1958).

The identification of the two *P. sojae* isolates (PS-15-TF3 and PS-14-F14) was confirmed using the internal transcribed spacer (ITS) regions of ribosomal DNA (Grünwald et al. 2011). DNA was extracted from the lyophilized mycelia of the two isolates grown in diluted V8 juice broth using the Wizard Genomic DNA Purification Kit (Promega Inc., Madison, WI). The internal transcribed spacer (ITS) region of the DNA was amplified using ITS4 and ITS6 primers (Grünwald et al. 2011). Reactions for the PCR

amplifications were performed in a 20 µl mixture containing approximately 1-3 ng/µl of DNA, 400 nM of each the forward and reverse primers, 2 mM of each dNTPs, 5 units/µl of Taq DNA Polymerase (Qiagen, Valencia, CA), and 10x Taq Buffer containing 15 mM $MgCl_2$ (Qiagen, Valencia, CA). The PCR parameters included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (Grünwald et al. 2011). To confirm amplification, a 7 µl aliquot of both PCR products was run on an agarose gel (2%). The PCR products were sequenced by Functional Bioscience Inc. (Madison, WI). Analysis of the edited ITS sequences of the two *P. sojae* isolates was performed using Basic Local Alignment Search Tool nucleotide (BLASTN) at GenBank nucleotide database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The two isolates were identified as *P. sojae* in the BLASTN searches based on lowest e-value (<10), highest score, and greatest similarity (>95%).

For the pathotype determination of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14) the hypocotyl inoculation technique was adopted on a set of 13 soybean differentials (Dorrance et al. 2008) with each differential having one specific *Rps* gene. The 13 differentials used in this study were obtained from the USDA-ARS Soybean Germplasm Collection, Ohio State/OARDC and these included Harlon (*Rps*1a), Harosoy 13XX (*Rps*1b), Williams 79 (*Rps*1c), PI 103091(*Rps* 1d),Williams 82 (*Rps*1k), L76-1988 (*Rps*2*)*, L83-570 (*Rps*3a), PRX-146-36 (*Rps*3b), PRX-145-48 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Haro 62xx (*Rps*6), Harosoy (*Rps*7), PI 399073 (*Rps*8) (Dorrance et al. 2004).The soybean cv. Williams was used a susceptible check. Fifteen seeds of 13 soybean

differentials and cv. Williams were sown in each styrofoam cup (473 ml) and grown for 7 days at 25-28ºC under 16 h photoperiod with a light intensity of 1000 $\mu Em^{-2}s^{-1}$ in the greenhouse. During the 7 days, the plants were watered daily. To inoculate the differentials for pathotyping the two *P. sojae* isolates, a slurry was prepared from a 2-week-old culture of *P. sojae* grown on lima bean agar (LBA; 100 ml lima bean broth and 12 g agar in 1000 ml distilled water). About 0.2 to 0.4 ml (approximately 200 to 400 cfu/ml) of the culture slurry was placed into the slit (1 cm) of the seedlings hypocotyl region with the help of the syringe (10 ml). After inoculation, the plants were incubated in a dew chamber (95% humidity) for 24 hat a temperature range of 20 to 22°C in the dark. After 24 h of incubation, the soybean plants were placed in a greenhouse at temperatures ranging from 22 to 28°C under natural light. Five to seven days after inoculation, the incidence of Phytophthora root rot was evaluated. The differential was considered susceptible when at least 7 of the 10 seedlings developed an expanding necrotic brown lesion. A differential was considered resistant if 70% or more of the plant inoculated with *P. sojae* survived (Dorrance et al. 2008). Based on the reaction of *P. sojae* isolates on the soybean differential, the Octal Code was determined with HaGiS spread sheet as described by Herrmann et al. (1999).

SCN extraction and inoculum

For SCN inoculum, eggs of *H. glycines* were recovered from a soil sample collected from Clay County, SD and the population was determined to be HG type 0 in a study conducted by Acharya et al. (2016). In this study, *H. glycines* HG type 0 was used because it was identified as the most common HG type on soybean in South Dakota by Acharya et al. (2016).

For the interaction study, in order to increase SCN population, a SCN susceptible cv. Williams 82 was used. Briefly, cysts of HG type 0 were collected in a 50 ml beaker using the method described by Faghihi et al. (1986). Cysts were crushed and SCN eggs were released from cysts with a stopper–bit assembly (Faghihi and Ferris 2000). The nematode inoculum was prepared in a water suspension with a density of 2,000 eggs and juveniles per ml by counting SCN eggs and juveniles using a nematode counting slide under a dissecting microscope at 40X magnification (Nikon SMZ745T, Nikon Instruments, Canada).

Interaction between *P. sojae* and SCN

For the interaction study between *P. sojae* and SCN, the experiment was set up in a completely randomized design in a factorial arrangement for the two *P. sojae* isolates, PS-15-TF3 and PS-14-F14, in the greenhouse. The experimental factors were cultivar treatment (four cultivars: Jack, Surge, Williams 82, and Williams), SCN treatment, and *P. sojae* treatment. The four soybean cultivars differed in their resistance to SCN and *P. sojae* (Jack is resistant to SCN and has *Rps*2 gene conferring tolerance to *P. sojae* ; Surge has *Rps*1 gene conferring tolerance to *P. sojae* ; Williams 82 is SCN susceptible and has *Rps*1k gene conferring tolerance to *P. sojae* ; Williams is susceptible to SCN and susceptible to *P. sojae*). For each *P. sojae* isolate, there were 3 treatments (SCN only, *P. sojae* only, and concomitant inoculation of SCN and *P. sojae*) and 5 replicates per treatment on all 4 soybean cultivars. Each plant in a cone-tainer was regarded as a replication. The experiment was performed twice for the two *P. sojae* isolates.

Before planting in 164 ml cone-tainers (Stuewe and Sons Inc., Tangent, OR), the seeds of the 4 soybean cultivars were pre-germinated in Petri dishes for 3 days. For each

cultivar, a total of 30 cone-tainers were filled with 80 g of steam-pasteurized sand: clay (2:1) soil mixture. Two agar plugs (5 mm diameter) from 10 day old LBA cultures of *P. sojae* were placed on either sides of the pre-germinated soybean seeds at a distance of 10 mm (Adeniji et al. 1975). The *P. sojae* inoculum was covered with 20 g of the steam pasteurized sand: clay mixture (2:1). After inoculating the soybean plants with either of the *P. sojae* isolates, the plants were transferred into a misting chamber for 48 h before SCN inoculation. After 48 h, a 25 mm deep hole was carefully made close to the soybean seedlings in each of the cone-tainers needing SCN treatment using a glass rod and 1 ml of the SCN suspension (containing 2000 eggs and juveniles) were added to the holes (Adeniji et al. 1975). The cone-tainers were placed in buckets filled with sand and maintained in a water bath at 26 ± 2°C in the greenhouse, with natural light supplements with a photoperiod of 16 h of artificial light for 35 days. The relative humidity in the greenhouse was maintained at 95% and air temperature was set at 22 to 25^{o}C.

After 35 days, to confirm pathogenicity of *P. sojae*, infected roots of random soybean plants representing *P. sojae* treatments (*P. sojae* only and concomitant inoculation of SCN and *P. sojae*) were sectioned longitudinally (approximately 1 cm length), surface-sterilized and placed on LBA. Plates were incubated at 22°C for 2 to 3 days in the dark and cultures were scored for presence or absence of *P. sojae* based on morphology (Jackson et al. 2004).

Data collection and analysis

At 35 days after SCN inoculation, data was collected on stem length, root length, fresh plant weight, fresh root weight, lesion length produced by *P. sojae* on soybean roots, number of SCN eggs and juveniles per plant for each treatment. For the two isolates, lesion

length caused by *P. sojae* was measured from the site of seed attachment to the end of the soybean roots where the lesion would have extended on each soybean seedling (Modified from Mideros et al. 2007). The SCN eggs and juveniles were counted using a nematode counting slide under a dissecting microscope at 40X magnification (Nikon SMZ745T, Nikon Instruments, Canada).

To determine whether the interaction between SCN and *P. sojae* can affect soybean growth, the relationship between soybean cultivars, *P. sojae* and SCN was analyzed using the linear mixed effects models in R (R core team 2012) using the *lme4* package (Bates et al. 2012). For the model, the variables "cultivar", "*P. sojae* infestation" (infected soybean roots or not) and "SCN infestation" (infested soybean roots or not) were entered as fixed effects. As random effects, "experimental repeat" and "replication" were included into the model. For the two *P. sojae* isolates, data was analyzed separately.

To determine the effect of *P. sojae* on SCN or the effect of SCN on *P. sojae* , *P. sojae* and SCN infestation was analyzed using the linear mixed effects models in R (R core team 2012) using the *lme4* package (Bates et al. 2012). For the model, the variables "cultivar" and "treatment" (*P. sojae* alone, SCN alone and combination of SCN and *P. sojae*) were entered as fixed effects. As random effects, "experimental repeat" and "replication" were included into the model. For the two *P. sojae* isolates, data was analyzed separately. For each isolate, the lesion length caused by *P. sojae* and SCN egg counts were subjected to analysis of variance (ANOVA) for a completely randomized design in R (v2.11.1; https://www.rstudio.com/) and treatment means were separated using Fisher's LSD test ($P \leq 0.05$) in the Agricolae package (de Mendiburu 2014).

For all analyses, the ANOVA assumptions of normality and homogeneity of variances were checked and satisfied before combining the results of the two experimental repeats. The *P*-values associated with the growth variables (stem length, root length, fresh root weight, fresh plant weight, lesion length and SCN count) was determined using the likelihood ratio test [in the *lme4* package (Bates et al. 2012)] in which a "full" model containing fixed effects and random effects was compared against a "reduced" model with only random effects. For the likelihood ratio test, the fixed effects were considered significant if the difference between the likelihood of the full model and reduced model was significant at $P \leq 0.05$.

Results

***Phytophthora sojae* isolation, identification and pathotype characterization**

For the two isolates, *P. sojae* grew on PBNIC agar media appearing dense white mycelium on the plates after 2 or 3 days and covering the whole plates within 7 to 10 days. The mycelium of *P. sojae* appeared to be coenocytic, highly branched with curved tips on PBNIC media plates. The color of the hyphae was white and branched mostly at right angles (Jackson et al. 2004). Oospores were formed on LBA within 3 to 4 days for the two isolates.

For molecular confirmation of *P. sojae*, approximately 850 bp of the ITS region was amplified from the two *P. sojae* isolates and used to query the GenBank database. A BLASTN search matched the ITS sequence of the *P. sojae* isolates with the ITS sequence of *Phytophthora sojae* strain ATCC MYA-3899 (Accession # FJ746643) with identities = 837/838 (99%) and gaps = 0/838 (0%). The ITS sequences of the *P. sojae isolates* (PS-15-

TF3 and PS-14-F14) generated in this study are deposited in the GenBank under accession numbers KX668417 and KX668418.

The *P. sojae* isolate PS-15-TF3 showed susceptible reaction to all the 13 soybean differentials (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, *Rps*2, *Rps*3a, *Rps*3b, *Rps*3c,*Rps*4, *Rps*5, *Rps*6 and *Rps*7) and is represented by virulence formula 77771.The *P. sojae* isolate PS-14-F14 showed susceptible reaction to only one soybean differential (*Rps*7) and is represented by virulence formula 00001(formally Race 1).

Interaction between *P. sojae* and SCN

Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as cultivar and treatment ($P > 0.05$). In the greenhouse, all soybean plants inoculated with *P. sojae* resulted in disease 35 days after SCN inoculation, and inoculated plants developed lesions on the roots. For all *P. sojae* treatments for the two isolates, the pathogen was isolated from the infected roots. *Phytophthora sojae* was not isolated from the soybean plants representing treatment with SCN only and the soybean plants with no infestation.

***P. sojae* isolate PS-15-TF3:** Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as cultivar, *P. sojae* or SCN ($P>0.05$). A significant three-way cultivar x SCN x *P. sojae* interaction was observed to affect the stem length (χ^2 =151.7, df =11, $P<0.001$), root length (χ^2 =385.6, df =11, $P<0.001$), fresh plant weight (χ^2 =83.5, df =11, $P<0.001$) and fresh root weight (χ^2 =35.6, df =11, P <0.001) of the soybean plants (Table 4.1). In addition, a significant two-way cultivar x SCN interaction ($P<0.001$), cultivar x *P. sojae* interaction ($P<0.001$) and *P. sojae* x SCN interaction ($P<0.001$) was observed affecting

stem length, root length, fresh plant weight and fresh root weight. While cultivar and *P. sojae* significantly affected all variables ($P<0.001$), SCN significantly affected only root length ($P=0.01$) and fresh plant weight ($P=0.02$).

Stem length was reduced in presence of both the pathogens by 2% for cv. Jack (LSD=12.0, $P= 0.28$), 4% for cv. Surge (LSD=18.2, $P= 0.61$) and 1% for cv. Williams 82 (LSD=12.2, $P= 0.35$) as compared to *P. sojae* treatment alone. However, significant differences in stem length were not observed among treatments for any of the four cultivars (Table 4.2, Table 4.3 and Table 4.4). On cv. Williams, stem length was reduced by 4% (LSD=14.5, $P= 0.03$) as compared to *P. sojae* treatment alone, although significant differences were not observed (Table 4.5).

Root length was reduced by 4% (LSD=14.4, $P= 0.61$) and 5% (LSD=25.2, $P= 0.51$) respectively as compared to *P. sojae* treatment alone for cv. Jack and cv. Williams (Table 4.2 and Table 4.5). However, significant differences in root length were not observed among treatments for the two cultivars. On cv. Surge and cv. Williams 82, root length was significantly reduced by 12% (LSD=21.8, $P= 0.04$) and 8% (LSD=19.7, $P=0.04$) respectively when infected by both the pathogens as compared to *P. sojae* treatment alone (Table 4.3 and Table 4.4).

Fresh plant weight was significantly reduced by 28% on cv. Jack (LSD=0.60, $P= 0.001$) when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.2). On cv. Surge, the reduction in fresh plant weight was 17% (LSD=0.81, $P= 0.34$) when co-infested with both the pathogens as compared to *P. sojae* infestation, however significant differences were not observed (Table 4.3). On cv. Williams 82, there were no significant differences in plant weight (LSD=0.58, $P= 0.20$) when infected by *P. sojae* and

SCN as compared to *P. sojae* treatment alone (Table 4.4). On cv. Williams, fresh plant weight was reduced by 6% (LSD=0.61, *P*= 0.05) when co-infected by both the pathogens as compared to *P. sojae* infestation only though statistically significant differences were not observed among treatments (Table 4.5).

Fresh root weight was significantly reduced by 26% (LSD=0.3, *P*=0.03) in presence of both the pathogen treatment as compared to *P. sojae* treatment only on cv. Jack (Table 4.2). On cv. Surge (LSD=0.41, *P*= 0.57) and cv. Williams 82 (LSD=0.21, *P*= 0.11), fresh root weight was reduced by 13% in presence of both the pathogen treatment as compared to *P. sojae* treatment only, however significant differences were not observed between the two treatments (Table 4.3 and Table 4.4). On cv. Williams, fresh root weight was reduced by 8% (LSD=0.33, *P*= 0.05) when the plants were infected by both *P. sojae* and SCN as compared to *P. sojae* treatment alone but significant differences were not observed between the two treatments (Table 4.5).

***P. sojae* isolate PS-14-F14:** A significant three way cultivar x SCN x *P. sojae* interaction was observed to affect all the growth parameters [stem length (χ^2 =116.4, df =11, *P*<0.001), root length (χ^2=48.5,df =11, *P*<0.001), fresh plant weight (χ^2 =51.2,df =11, *P*<0.001)] except for fresh root weight (χ^2=14.0, df=11, *P*= 0.23) (Table 4.1). In addition, a significant two-way cultivar x SCN interaction (*P*<0.001) affected stem length. A significant *P. sojae* x SCN interaction (*P*<0.001) affected stem length, root length, and fresh plant weight. While *P. sojae* infection significantly affected only stem length (*P*=0.03) and SCN significantly affected stem length (*P*<0.001), fresh plant weight (*P*<0.001) and fresh root weight (*P*=0.005), cultivar significantly affected all variables except root length (*P*<0.001).

Stem length was reduced by 4% (LSD=12.3, *P*= 0.26) when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone on cv. Jack (Table 4.2). On cv. Surge, stem length was reduced by 2% (LSD=17.6, *P*= 0.54) when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.3). However, on cv. Jack and cv. Surge, significant differences in stem length were not observed among treatments. On Williams 82, stem length was significantly reduced by 8% (LSD=14.45, *P*= 0.03) when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.4). On cv. Williams, the stem length was reduced by 4% (LSD=13.9 *P*= 0.69) in both pathogen treatment as compared to *P. sojae* treatment alone but there were no statistical differences (Table 4.5).

Root length was reduced by 2% and 5% when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone on cv. Jack (LSD=12.4, *P*= 0.24) and cv. Williams 82 (LSD=0.60, *P*= 0.78), respectively (Table 4.2 and Table 4.4). On cv. Surge and cv. Williams, root length was reduced by 4% (LSD=20.8, *P*= 0.46) and 2% (LSD=18.6, *P*= 0.45) respectively in presence of both the pathogens as compared to *P. sojae* treatment alone (Table 4.3 and Table 4.5). However, significant differences in root length were not observed between co-infection of soybean plants by *P. sojae* and SCN as compared to *P. sojae* by itself for any of the cultivars.

Fresh plant weight was significantly reduced by 15% (LSD=0.69, *P*= 0.02) on cv. Jack when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.2). On cv. Williams 82, plant weight reduced by 6% (LSD=0.60, *P*= 0.19) between when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone, but there were no significant differences (Table 4.4) On cv. Williams and cv. Surge, fresh plant weight was reduced by 13% (LSD=0.51, *P*= 0.46) and 10% (LSD=0.73, *P*= 0.22) respectively, in

presence of both the pathogens as compared to *P. sojae* treatment alone (Table 4.3 and Table 4.5). However, significant differences in root length were not observed between the two treatments for these two cultivars.

Fresh root weight was reduced by 11% (LSD=0.22, *P*= 0.19) and 6% (LSD=0.29, *P*= 0.23) on cv. Williams 82 and cv. Jack respectively when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.2 and Table 4.4). On cv. Surge, fresh root weight was reduced in presence of both the pathogens by 12% (LSD=0.34, *P*= 0.46) as compared to *P. sojae* treatment alone (Table 4.3). On cv. Williams, fresh root weight was reduced by 6% (LSD=0.29, *P*= 0.63) when infected by *P. sojae* and SCN as compared to *P. sojae* treatment alone (Table 4.5). However, for any of the cultivars, significant differences in root length were not observed between co-infection of soybean plants by *P. sojae* and SCN as compared to *P. sojae* by itself.

Effect of SCN on *P. sojae*

Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as cultivar and treatment (*P*> 0.05).

***P. sojae* isolate PS-15-TF3**: A significant two-way cultivar x treatment interaction was observed to affect the lesion length caused by *P. sojae* on soybean (χ^2=176.5, df=6, *P*<0.001); therefore lesion length data obtained for each cultivar were analyzed separately. On cv. Jack, the lesion length caused by PS-15-TF3 on soybean plants was significantly higher by 23% (LSD=4.6, *P*<0.001) in the presence of SCN as compared to the *P. sojae* isolate by itself (Table 4.2). On cv. Surge, lesion length was significantly increased by 15% (LSD= 4.4, *P*<0.001) in the presence of SCN (Table 4.3). On cv. Williams 82, the lesion

length was significantly increased (LSD=5.3, $P<0.001$) by 10% in the presence of SCN (Table 4.4). On cv. Williams, lesion length produced by PS-15-TF3 was significantly increased by 8% (LSD= 4.3, $P<0.001$) rise in the presence of SCN as compared to the *P. sojae* isolate by itself (Table 4.5).

***P. sojae* isolate PS-14-F14:** A significant two-way cultivar x treatment interaction was observed to affect the lesion length caused by *P. sojae* on soybean ($\chi^2=104.16$, df =6, $P<0.001$); therefore lesion length data obtained for each cultivar were analyzed separately. On cv. Jack (LSD=6.3, $P= 0.05$), the lesion length caused by PS-14-F14 on soybean plants was higher by 14% in the presence of SCN as compared to the *P. sojae* isolate by itself (Table 4.2), although significant differences were not observed between the two treatments. On cv. Surge (LSD=5.7, $P= 0.76$), lesion length caused by PS-14-F14 was increased by 2% in the presence of SCN but it was not significantly different from that caused by the treatment with only *P. sojae* (Table 4.3). On cv. Williams 82 (LSD=3.9, $P= 0.51$), although significant differences were not observed when compared with *P. sojae* by itself, the lesion length caused by PS-14-F14 was increased by 5% in the presence of SCN (Table 4.4). On cv. Williams (LSD=4.6, $P= 0.10$), the lesion length caused by PS-14-F14 was increased by 8% on soybean plants in the presence of SCN as compared to the *P. sojae* by itself (Table 4.5).

Effect of *P. sojae* on SCN

Test statistics indicated there was no significant effect of experiment or interaction effects between experiment and other experimental factors such as cultivar and treatment ($P>0.05$).

***P. sojae* isolate PS-15-TF3**: A significant two-way cultivar x treatment interaction was observed to affect the SCN population on soybean plants ($\chi^2=4.5$, df =1, $P=0.033$); therefore data obtained for SCN egg number was analyzed separately for each cultivar. On cv. Jack, the number of SCN eggs and juveniles were significantly reduced by 18% (LSD=106.0, $P=0.025$) in the presence of *P. sojae*, as compared to SCN treatment only. On cv. Surge, the number of SCN eggs and juveniles were significantly reduced by 50% (LSD=1813.2, $P<0.001$) in the presence of *P. sojae* as compared to soybean plants inoculated with SCN only (Table 4.6). On cv. William 82, SCN population was significantly reduced by 72% (LSD=4423.3, $P<0.001$) in soybean plants co-infected by SCN and *P. sojae* as compared to SCN treatment only (Table 4.6). On cv. Williams, although no statistical differences were observed, SCN population reduced by 16% (LSD=446.7, $P=0.06$) when co-inoculated with PS-15-TF3 as compared to when soybean plants inoculated with SCN treatment only (Table 4.6).

***P. sojae* isolate PS-14-F14**: A significant two-way cultivar x treatment interaction was observed to affect the SCN population on soybean seedlings (χ^2 =194.9, df =6, $P<0.001$); therefore data obtained for SCN egg number was analyzed separately for each cultivar. On cv. Jack, although there were no significant differences, the SCN numbers were reduced by 5% (LSD=86.3, $P=0.37$) in the presence of *P. sojae* (Table 4.6). On cv. Surge, the number of SCN eggs and juveniles were significantly reduced by 69% (LSD=1163.9, $P<0.001$) in the presence of *P. sojae* as compared to soybean plants

inoculated with SCN only (Table 6). On cv. William 82, the number of SCN eggs and juveniles were significantly reduced by 47% (LSD=4815.9, $P<0.001$) in the presence of *P. sojae* as compared to soybean plants inoculated with SCN only (Table 4.6). On soybean cv. Williams, although no significant differences observed, SCN population was reduced by 8% (LSD=493.0, $P=0.33$) in the presence of *P. sojae* as compared to when soybean plants inoculated with SCN only (Table 4.6).

Discussion

This study examined the differences in interaction between two pathotypes of *P. sojae* and SCN on soybean in the greenhouse. In this study, *P. sojae* isolates and SCN had damaging effect on all the growth variables of the soybean plants in the combined presence of both the pathogens as compared to single pathogen treatment, however the effect was more when infested with *P. sojae* isolate PS-15-TF3. Irrespective of the host genetics, lesion length caused by *P. sojae* isolate PS-15-TF3 was higher on all the four soybean cultivars in the presence of SCN as compared to lesion length caused by *P. sojae* isolate PS-15-TF3 and PS-14-F14 alone. In contrast, SCN population was reduced when the soybean plants were co-infested with SCN and either of the two *P. sojae* isolates (PS-15-TF3 and PS-14-F14) as compared to SCN treatments.

While studying the effect of interaction between *P. sojae* and SCN on soybean growth, differences in the growth variables were observed between the two *P. sojae* isolates on all the four cultivars (Tables 4.2-4.5). For example, *P. sojae* affected stem length, root length, fresh plant weight and fresh root weight of all cultivars when inoculated with PS-15-TF3. For PS-14-F14, *P. sojae* affected only stem length of the soybean plants across all cultivars (Table 4.1).Similar observations were reported by Mideros et al. (2007), when

two pathotypes OHR1 (virulent on differentials with *Rps*7 gene) and 1.S.1.1 (virulent on differentials with *Rps*1a, *Rps*1b, *Rps*1k, *Rps*2, *Rps*3a, *Rps*3b, *Rps*3c, *Rps*4, *Rps*5, *Rps*6, *Rps*7, and *Rps*8 genes), were inoculated on 8 genotypes with varying levels of partial resistance. In the study by Mideros et al. (2007), a significant isolate x host genotype interaction was observed for lesion length, infection frequency and number of oospores and it was speculated that the interaction was observed due to "isolate-specific resistance genes" since the two isolates varied in their virulence on the 8 genotypes. In our study, the three cultivars used had "isolate-specific resistance genes" only for PS-14-F14 and the effect of PS-14-F14 on growth variables was lower as compared to PS-15-TF3. However, irrespective of whether the soybean cultivars had isolate-specific resistance genes, we observed that the soybean growth variables were greatly affected as a result of the co-infestation of the plants by the two pathogens as compared to infection by *P. sojae* alone. Adeniji et al. (1975) reported similar observations that the shoot and root weight of three soybean cultivars (Carosoy, Dyer and Harosoy-63) was lower when inoculated in combination with SCN compared to inoculated with *P. sojae* alone but differences were not significant.

While determining the effect of SCN on *P. sojae*, it was determined that an increase in lesion length caused by *P. sojae* was observed for the two isolates on the four soybean cultivars in the presence of SCN, when the disease was assessed on soybean plants 35 days after inoculation. Previous research on fungal-nematode interactions have shown that nematodes can wound plant roots and break-down resistance in crop plants as a result of which the plants can become susceptible to fungal pathogens (Ragozzino and d'Errico 2011). For example, greenhouse trials were conducted by Diaz Arias (2012) to determine

whether SCN infestation enhances root rot caused by species of *Fusarium* on soybean by using cultivars differing in genetic resistance to SCN. Two isolates from each of 8 *Fusarium* species were tested on root rot severity, number of SCN females, and root morphological characteristics. Depending on the *Fusarium* isolates and species, enhanced root rot severity and root damage was observed when SCN was combined with the *Fusarium* isolates as compared to single pathogen treatment. In general, *P. sojae* is managed by use of race-specific resistance (single *Rps* gene) and partial resistance containing multiple genes (Sugimoto et al. 2012). However, in South Dakota, there is an increased prevalence of Phytophthora stem and root rot of soybean and it is unclear if SCN has any role in increasing the susceptibility of partially resistant soybean cultivars to *P. sojae*. Among the two *P. sojae* pathotypes, PS-15-TF3 was virulent on all 13 *Rps* differentials (R. N. Chowdhury et al. *unpublished*), and none of the four cultivars used in this study have resistance to this pathotype. Therefore, it might be speculated that lesion length caused by *P. sojae* increased in the presence of SCN as compared to *P. sojae* treatment by itself because PS-15-TF3 is able to overcome the partial resistance in the three cultivars (Jack, Surge and Williams 82). However, for PS-14-F14, the lesion length caused by *P. sojae* increased in the presence of SCN on cv. Jack, cv. Williams 82 and cv. Surge as compared to the pathogen by itself despite that the three cultivars had partial resistance to PS-14-F14. Similar observations with regards to increased lesion length by *P. sojae* in the presence of SCN were made by Adeniji et al. (1975) and Kaitany et al. (2000) in the interaction study between the two pathogens. They hypothesized that SCN may be involved in modifying the physiology of soybean thus increasing the susceptibility of the plants to infection by *P. sojae*.

While studying the effect of *P. sojae* on SCN, it was observed that SCN population was significantly reduced on all the four soybean cultivars in the presence of the two *P. sojae* isolates. In general, the ability of SCN to reproduce on soybean roots can be affected when the nematode cannot obtain nutrients from the host or cannot sustain feeding on the host because of the changes in host's defense mechanism (McCarville et al. 2014). In this study, a decrease in SCN population was observed on the soybean plants possibly because the roots were already colonized by *P. sojae* as a result of which the root mass and food base for SCN feeding was reduced (Adeniji et al. 1975). Moreover, *P. sojae* is known to produce toxic metabolites during the formation of sporangium that may affect the reproduction of SCN (Jing-zhi et al. 2012). For example, in a study by Dong et al (2012), the expression of NLP protein (24-kDa protein that induces cell death and ethylene accumulation) in *P. sojae* was studied and it was shown that 20 of the NLP proteins were highly expressed during cyst germination and infection stages. Although the toxins produced by *P. sojae* was not explored in this study, it may be speculated that toxic metabolites produced by *P. sojae* may have affected the reproduction of SCN on soybean.

In summary, our study provides insight into the possible interaction between SCN and *P. sojae* on soybean under controlled conditions. Our results show that SCN and *P. sojae* interact additively thus compromising the overall growth variables of the soybean plants irrespective of the nature of virulence pathotypes. In general, interaction between multiple pests on soybean can lead to higher yield losses under field conditions. For example, field studies were conducted by Diaz Arias (2012) on the interaction between SCN and Fusarium root rot species affecting root rot severity and they found enhanced yield losses in the combined presence of SCN and *Fusarium* as compared to single

pathogen treatment. For this study, we have not tested the effect of interaction between *P. sojae* and SCN on soybean under field conditions. However, it is possible that yield and other agronomic factors can be compromised as a result of the interaction between the two pathogens. Currently, *P. sojae* and SCN are managed using integrated pest (disease) management approaches such as selecting soybean varieties with tolerance to *P. sojae* and resistance to SCN, seed treatments and crop rotation. Based on our results, use of only partially resistant *P. sojae* soybean cultivars cannot protect the crop from *P. sojae* because infection of soybean plants by *P. sojae* may be exacerbated by SCN irrespective of the nature of pathotypes that exist in the farmers' field. However, if the soybean farmers use cultivars with resistance to SCN and partial resistance to *P. sojae*, it is possible to manage the disease complex caused by the two pathogens and protect yield in their fields.

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Table 4.1 *P* values for main and interaction effect of cultivars (Jack, Surge, William 82, and Williams) and pathogen treatments (SCN, *P. sojae* isolates PS-15-TF3 and PS-14-F14 or concomitant inoculations of the two pathogens) on soybean growth.

	Effects[a,b,c,d]							
***P. sojae* isolates**	Variables	Cultivar	SCN	*P. sojae*	Cultivar x SCN	Cultivar x *P. sojae*	SCNx *P. sojae*	Cultivar x SCN x *P. sojae*
PS-15-TF3	Stem length	<0.001	ns	0.008	0.02	ns	<0.001	<0.001
	Root length	<0.001	0.01	<0.001	<0.001	ns	<0.001	<0.001
	Fresh plant weight	<0.001	0.02	0.001	0.003	0.03	<0.001	<0.001
	Fresh root weight	<0.001	ns	0.002	0.014	ns	<0.001	<0.001
PS-14-F14	Stem length	<0.001	0.03	0.004	0.025	ns	<0.001	<0.001
	Root length	<0.001	ns	ns	ns	ns	<0.001	<0.001
	Fresh plant weight	<0.001	<0.001	ns	ns	ns	<0.001	<0.001
	Fresh root weight	ns	0.005	ns	ns	ns	ns	ns

[a]*P*-values associated with growth variables (stem length, root length, fresh root weight, and fresh plant weight) was determined using the likelihood ratio test [in the *lme4* (Bates et al. 2012) package] in which a "full" model containing fixed effects was compared against a "reduced" model without the fixed effects. For the likelihood ratio test, the fixed effect was considered significant if the difference between the likelihood of the full and reduced models was significant at $P \leq 0.05$.

[b]Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R.

[c]ns=not significant at $P \geq 0.05$.

[d]Abbreviation: SCN=Soybean Cyst Nematode

Table 4.2 Shoot, root and lesion length measurements observed on the soybean cv. Jack inoculated with SCN or concomitant inoculations of SCN with either of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14)

***P. sojae* isolates**	**Treatments[a,b,c]**	**Stem length (mm)[a]**	**Root length (mm)[a]**	**Fresh plant weight (g)[a]**	**Fresh root weight (g)[a]**	**Lesion length (mm)[d]**
PS-15-TF3	SCN	164.3 a	208.7 a	3.2 b	1.7 ab	N/A
	PS-15-TF3	173.7 a	214.3 a	4.1 a	1.9 a	60.6 b
	PS-15-TF3 +SCN	162.5 a	205.0 a	3.2 b	1.5 b	78.7 a
PS-14-F14	SCN	164.3 a	208.7 a	3.2 a	1.7 a	N/A
	PS-14-F14	165.6 a	217.5 a	3.8 a	1.7 a	36.9 a
	PS-14-F14+SCN	158.7 a	213.1 a	3.3 a	1.6 a	43.1 a

[a] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R. Values are the means of two experiments with a total of ten replications each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P \leq 0.05$).

[b] Treatments involving PS-15-TF3 include those treatments that were inoculated with only PS-15-TF3(five replications) and concomitant inoculation with SCN (five replications).

[c] Treatments involving PS-14-F14 include those treatments that were inoculated with only PS-14-F14 (five replications) and concomitant inoculation with SCN (five replications).

[d] Lesion length caused by *P. sojae* was measured from the site of seed attachment to the end of the soybean roots where the lesion would have extended on each soybean seedling (Modified from Mideros et al. 2007).On the SCN control, no lesion was observed on the soybean roots and no pathogen was recovered.

Table 4.3 Shoot, root and lesion length measurements observed on the soybean cv. Surge inoculated with SCN or concomitant inoculations of SCN with either of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14)

***P. sojae* isolates**	**Treatments[a,b,c]**	**Stem length (mm)[a]**	**Root length (mm)[a]**	**Fresh plant weight (g)[a]**	**Fresh root weight (g)[a]**	**Lesion length (mm)[d]**
PS-15-TF3	SCN	181.2 a	225.0 a	3.4 a	1.7 a	N/A
	PS-15-TF3	180.0 a	226.7 a	3.2 a	1.7 a	35.0 b
	PS-15-TF3 +SCN	173.1 a	201.8 b	2.9 a	1.5 a	41.2 a
PS-14-F14	SCN	181.2 a	225.0 a	3.4 a	1.7 a	N/A
	PS-14-F14	175.6 a	220.0 a	2.8 a	1.8 a	37.5 a
	PS-14-F14+SCN	171.8 a	212.5 a	2.8 a	1.6 a	38.1 a

[a] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R. Values are the means of two experiments with a total of ten replications each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P \leq 0.05$).

[b] Treatments involving PS-15-TF3 include those treatments that were inoculated with only PS-15-TF3(five replications) and concomitant inoculation with SCN (five replications).

[c]Treatments involving PS-14-F14 include those treatments that were inoculated with only PS-14-F14 (five replications) and concomitant inoculation with SCN (five replications).

[d] Lesion length caused by *P. sojae* was measured from the site of seed attachment to the end of the soybean rootswhere the lesion would have extended on each soybean seedling (Modified from Mideros et al. 2007).On the SCN control, no lesion was observed on the soybean roots and no pathogen was recovered.

Table 4.4 Shoot, root and lesion length measurements observed on the soybean cv. William 82 inoculated with SCN or concomitant inoculations of SCN with either of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14)

***P. sojae* isolates**	**Treatments[a,b,c]**	**Stem length (mm)[a]**	**Root length (mm)[a]**	**Fresh plant weight (g)[a]**	**Fresh root weight (g)[a]**	**Lesion length (mm)[d]**
PS-15-TF3	SCN	185.6 a	208.7 a	3.3 a	1.9 a	N/A
	PS-15-TF3	178.9 a	199.2 a	2.6 b	1.7 a	63.1 b
	PS-15-TF3 +SCN	177.5 a	184.3 b	2.6 b	1.7 a	70.0 a
PS-14-F14	SCN	185.6 a	208.7 a	3.3 a	1.9 a	N/A
	PS-14-F14	180.0 ab	208.7 a	3.5 a	1.9 a	60.6 a
	PS-14-F14+SCN	166.2 b	198.7 a	3.3 a	1.7 a	64.1 a

[a] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R. Values are the means of two experiments with a total of ten replications each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P \leq 0.05$).

[b] Treatments involving PS-15-TF3 include those treatments that were inoculated with only PS-15-TF3(five replications) and concomitant inoculation with SCN (five replications).

[c] Treatments involving PS-14-F14 include those treatments that were inoculated with only PS-14-F14 (five replications) and concomitant inoculation with SCN (five replications).

[d] Lesion length caused by *P. sojae* was measured from the site of seed attachment to the end of the soybean roots where the lesion would have extended on each soybean seedling (Modified from Mideros et al. 2007). On the SCN control, no lesion was observed on the soybean roots and no pathogen was recovered.

Table 4.5 Shoot, root and lesion length measurements observed on the soybean cv. Williams inoculated with SCN or concomitant inoculations of SCN with either of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14)

***P. sojae* isolates**	**Treatments[a,b,c]**	**Stem length (mm)[a]**	**Root length (mm)[a]**	**Fresh plant weight (g)[a]**	**Fresh root weight (g)[a]**	**Lesion length (mm)[d]**
PS-15-TF3	SCN	130.0 a	195.0 a	2.3 a	1.7 a	N/A
	PS-15-TF3	115.0 b	191.2 a	1.7 b	1.3 b	75.6 b
	PS-15-TF3 +SCN	111.2 b	181.2 a	1.6 b	1.2 b	81.9 a
PS-14-F14	SCN	130.0 a	195.0 a	2.3 a	1.7 a	N/A
	PS-14-F14	130.0 a	187.5 a	2.5 a	1.8 a	36.6 a
	PS-14-F14+SCN	125.0 a	183.7 a	2.2 a	1.6 a	40.6 a

[a] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R. Values are the means of two experiments with a total of ten replications each. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P \leq 0.05$).

[b] Treatments involving PS-15-TF3 include those treatments that were inoculated with only PS-15-TF3(five replications) and concomitant inoculation with SCN (five replications).

[c]Treatments involving PS-14-F14 include those treatments that were inoculated with only PS-14-F14 (five replications) and concomitant inoculation with SCN (five replications).

[d] Lesion length caused by *P. sojae* was measured from the site of seed attachment to the end of the soybean roots where the lesion would have extended on each soybean seedling (Modified from Mideros et al. 2007).On the SCN control, no lesion was observed on the soybean roots and no pathogen was recovered.

Table 4.6. Mean number of SCN eggs (per gm of soybean root weight) on each of the four soybean cultivars from treatments inoculated with SCN or concomitant inoculations of SCN with either of the *P. sojae* isolates (PS-15-TF3 and PS-14-F14)

Cultivar	*P. sojae* isolates	Treatments[a,b]	SCN eggs (per gm of soybean root weight)[c,d]
Jack	PS-15-TF3	SCN	806.1 a
		PS-15-TF3 +SCN	682.7 b
	PS-14-F14	SCN	806.1 a
		PS-14-F14+SCN	768.9 a
Surge	PS-15-TF3	SCN	19680.2 a
		PS-15-TF3 +SCN	13116.2 b
	PS-14-F14	SCN	19680.2 a
		PS-14-F14+SCN	11577.4 b
Williams 82	PS-15-TF3	SCN	30811.5 a
		PS-15-TF3 +SCN	16044.8 b
	PS-14-F14	SCN	30811.5 a
		PS-14-F14+SCN	17730.2 b
Williams	PS-15-TF3	SCN	3081 a
		PS-15-TF3 +SCN	2660 a
	PS-14-F14	SCN	3081 a
		PS-14-F14+SCN	2853 a

[a] Treatments involving PS-15-TF3 include all those that were inoculated with SCN (five replications) and concomitant inoculation with SCN (five replications). The LSD analyses was performed by cultivar for treatments involving PS-15-TF3.

[b]Treatments involving PS-14-F14 include all those that were inoculated with SCN (five replications) and concomitant inoculation with SCN (five replications). The LSD analyses was performed by cultivar for treatments involving PS-14-F14.

[c] Analysis of variance was conducted by combining the data of two experimental repeats after testing for homogeneity of variance at $P \leq 0.05$ in R. Values are the means of two experiments with a total of ten replications each. Values within a column followed by the

same letter are not significantly different according to Fisher's least significant difference ($P \leq 0.05$).

[d] SCN eggs and juveniles were counted using a nematode counting slide under a dissecting microscope at 40X magnification (Nikon SMZ745T, Nikon Instruments, Canada). On the *P. sojae* control, no SCN eggs was observed under the microscope.

CHAPTER 5

General conclusions and recommendations

The main objectives of this research were to determine the pathotype diversity of *P. sojae* in commercial fields in South Dakota, to compare inoculation methods and determine new sources of partial resistance to *P. sojae* and to study the interaction of *P. sojae* with the soybean cyst nematode on soybean.

A three year survey (2013 to 2015) was conducted in South Dakota covering a total of 384 commercial soybean fields in 30 different counties and soil samples were randomly collected from each of the fields. Of 114 isolates that were recovered, 70 *P. sojae* isolates were evaluated for pathotype identification by using 13 differential cultivars each having single *Rps* gene and 50 different *P. sojae* pathotypes were identified. Our results suggest that at least 6 of the *Rps* genes were defeated by the 26% isolates of *P. sojae*, which indicates that the complexity of the isolates of *P. sojae* is continuing to increase in South Dakota.

We compared three greenhouse inoculation methods (inoculum layer test, tray test and rice grain inoculation) to identify a suitable inoculation method to screen soybean genotypes for partial resistance to *P. sojae.* Among the inoculation methods, highest recovery of *P. sojae* was observed for inoculum layer test (94.5%) inoculated with P. sojae isolate PS-15-TF3 compared to the other to two test (tray test and rice grain inoculation method) and the recovery was poorly negatively correlated with lesion length produced by the two *P. sojae* isolates at 7 days after inoculation on soybean plants (cv. Williams and cv. Surge) in independent experiments. Therefore, inoculum layer method was adopted for evaluation of partial resistance in 100 recombinant inbred line (RIL) population, which

were derived from the cross between cv. Surge and *Glycine soja*. The 100 RILs were evaluated for partial resistance to two isolates of *P. sojae* (PS-15-TF3 that is virulent on 13 differentials and PS-14-F14 that is virulent on differential carrying *Rps*7 gene) in the greenhouse. In the screening experiment, we identified 9 RILs that had relatively shorter lesion size as compared to the parents *Glycine soja* and cv. Surge.

We also examined the interaction between soybean cyst nematode (SCN) and *P. sojae* on soybean in the greenhouse. The interaction was examined on 4 cultivars (Jack, Surge, William 82 and Williams) with varying level of resistance and susceptibility to both *P. sojae* and SCN. Our results suggest that the combined presence of *P. sojae* and SCN affected the soybean growth variables irrespective of the nature of *P. sojae* pathotypes. Additionally, the lesion length caused by *P. sojae* was increased for the two isolates on the four soybean cultivars in the presence of SCN. However, SCN population was significantly reduced on all the four soybean cultivars in the presence of the *P. sojae* as compared to the SCN treatment.

Overall, the research presented in this thesis has advanced our understanding of *P. sojae* in South Dakota, which includes pathotype diversity, new sources of partial resistance to *P. sojae* and interaction with SCN on soybean. The *P. sojae* diversity results indicated the *Rps* genes often defeated and recommendations for management would be to use soybean cultivars with *Rps* genes that are not often defeated such as *Rps*2, 3a and 3b or use cultivars with sacked *Rps* genes. Given the pathotype diversity of *P. sojae* in South Dakota and the additive interaction of *P. sojae* with SCN, it was important to screen soybean germplasm (e.g. RILs used in this study) to identify new sources of resistance to the pathogen. The 9 RILs identified in this study can be potential sources of resistance to

P. sojae and can be used by a breeding program to development commercial soybean varieties with resistance to the pathogen. Additionally, the information generated in this research with regards to the pathotype diversity and interaction studies can be used for developing integrated pest management programs to manage *P. sojae* affecting soybean in South Dakota.